Nuclear Transport of 5S Ribosomal RNA in *Xenopus laevis* Oocytes

A thesis submitted in fulfilment
of the requirements for the degree of

Doctor of Philosophy

in
Cellular and Molecular Biology
at the
University of Canterbury

Kirstie J. Murdoch

1995

Table of Contents

Page

Abstract vi

Abbreviations vii

Chapter One. General Introduction 1

1.1 The Nuclear Membrane as a Selective Barrier 1

1.2 Movement of 5S rRNA Between the Nucleus and Cytoplasm 2

1.3 The Structure and Function of the Nuclear Pore Complex 5
1.3.1 Basic structure and function 5
1.3.2 Specific proteins of the NPC 6

1.4 Nuclear Transport Signals 8
1.4.1 Proteins 8
1.4.2 RNAs/RNPs 11

1.5 Nuclear Localisation Signal Receptors and Other *Trans*-acting Factors in Nuclear Transport 14
1.5.1 Saturability of nuclear transport 14
1.5.2 Characterisation of factors involved in the two steps of nuclear protein import 15
1.5.2.1 NLS binding and docking at the NPC 16
1.5.2.2 Translocation 17
1.5.3 *Trans*-acting factors in RNP nuclear transport 19

1.6 Shuttling Proteins 21

1.7 5S rRNA Nuclear Transport and the Scope of This Thesis 22

Chapter Two. 5S rRNA Nuclear Import: A Passive or Facilitated Process Through the Nuclear Pore Complex? 24

2.1 Introduction 24
2.1.1 Facilitated versus passive transport 24
2.1.2 Temperature dependence and ATP requirement 27
2.1.3 Wheat germ agglutinin 29
2.1.4 Assays used in the present study 31

2.2 Materials and Methods 32
2.2.1 *In vitro* synthesis of ^{32}P-5S rRNA 32
2.2.2 Surgical removal of oocytes and preparation for microinjection 33
2.2.3 Microinjection 34

2.2.4 Nuclear dissections and RNA extraction 34
2.2.5 Gel electrophoresis and autoradiography 35
2.2.6 ATP depletion, WGA and chilling assays 36
2.2.7 FITC-dextran controls 36

2.3 Results 37
2.3.1 ^{32}P-5S rRNA microinjected into the oocyte cytoplasm accumulates in the nucleus over time 37
2.3.2 Wheat germ agglutinin 37
2.3.3 Chilling 41
2.3.4 ATP depletion 44

2.4 Discussion 44
2.4.1 The nuclear accumulation of 5S rRNA 44
2.4.2 The effect of chilling, ATP depletion and WGA on 5S rRNA nuclear import 46
2.4.3 Facilitated transport of 5S rRNA 48

Chapter Three. A Role for Ribosomal Protein L5 in 5S rRNA Nuclear Transport 50

3.1 Introduction 50
3.1.1 The importance of RNA-protein interactions 50
3.1.2 Nuclear transport of RNPs 50
3.1.3 5S rRNA-protein interactions and nuclear transport 52
3.1.4 The study outlined in this chapter 54

3.2 Materials and Methods 55
3.2.1 *In vitro* synthesis of ^{35}S-5S RNPs 55
3.2.2 Microinjection and analysis of nuclear transport 56
3.2.3 Electrophoretic mobility shift assay for 5S RNPs 57

3.3 Results 58
3.3.1 *In vitro* synthesis and characterisation of ^{35}S-labelled *Xenopus* oocyte-type 5S rRNA-containing 5S RNPs 58
3.3.2 Comparison of the nuclear import kinetics of microinjected ^{35}S-5S RNPs with ^{32}P-5S rRNA 61
3.3.3 The effect of chilling on the nuclear import of ^{35}S-5S RNPs 63
3.3.4 The effect of oocyte ATP depletion on the nuclear import of ^{35}S-5S RNPs 63
3.3.5 The effect of wheat germ agglutinin on the nuclear import of ^{35}S-5S RNPs 65

3.4 Discussion 65
3.4.1 Protein binding and the compartmentalisation of 5S rRNA .. 66
3.4.2 Comparison of 5S rRNA and 5S RNP nuclear import 67
3.4.3 Facilitated transport of 5S RNPs 68
3.4.4 Summary 69

Chapter Four. Comparison of the 5S rRNA Nuclear Transport Pathway with that of Other Molecules 70

4.1 Introduction 70
4.1.1 The saturability of nuclear transport processes 70
4.1.2 Comparison of nuclear transport pathways for different molecules 72
4.1.3 Comparison of the pathway for 5S rRNA nuclear import with other molecules 75

4.2 Materials and Methods 76
4.2.1 Plasmids and proteins used in this study 76
4.2.2 High-yield synthesis of RNA 76
4.2.3 Preparation of RNA from oocytes and liquid scintillation counting 77

4.3 Results 78
4.3.1 The saturability of 5S rRNA nuclear transport 78
4.3.2 The effect of U1 snRNA on 5S rRNA nuclear transport ... 80
4.3.3 The effect of U3 snoRNA on 5S rRNA nuclear transport ... 80
4.3.4 The effect of the SV40 large T antigen nuclear localisation signal on 5S rRNA nuclear transport 82

4.4 Discussion 85
4.4.1 Requirements for saturation of the 5S rRNA/5S RNP import pathway 85
4.4.2 Signals and receptors required for 5S rRNA/5S RNP nuclear import 86
4.4.3 A role for shuttling proteins in 5S rRNA/5S RNP import? .. 87
4.4.4 Implications for coordinated nuclear import 89
4.4.5 Summary 90

Chapter Five. General Discussion 91

5.1 The Pathway for 5S rRNA Nuclear Import 91

5.2 Shuttling 93

5.3 Coordination of Ribosome Assembly 95

5.4 Prospects and Conclusions 96

Acknowledgements 98

References 99

Appendix 121

List of Figures

Page

2.1 The nuclear accumulation of microinjected oocyte-type ^{32}P-5S rRNA 38

2.2 The effect of WGA on the nuclear import of 5S rRNA ..... 39 & 40

2.3 The effect of chilling on the nuclear import of 5S rRNA ..... 42 & 43

2.4 The effect of ATP depletion on the nuclear import of 5S rRNA 45

3.1 Characterisation of *in vitro*-synthesised ^{35}S-5S RNPs 60

3.2 Nuclear accumulation of cytoplasmically microinjected ^{32}P-5S rRNA and ^{35}S-5S RNPs with time 62

3.3 The effect of chilling on the nuclear import of 5S RNPs 64

3.4 The effect of cellular ATP depletion on the nuclear import of 5S RNPs ... 64

3.4 The effect of WGA on the nuclear import of 5S RNPs 64

4.1 The saturability of 5S rRNA nuclear import 79

4.2 5S rRNA import in the presence of U1 snRNA 81

4.3 5S rRNA import in the presence of U3 snoRNA 83

4.4 5S rRNA import in the presence of P(lys)-BSA 84

5.1 Models for the pathway of nuclear import of 5S rRNA in *Xenopus* oocytes 94

List of Tables

Page

Table 1.1 Types of protein nuclear localisation signal 10

Table 1.2 Factors involved in nuclear protein import 19

Table 3.1 Proteins involved in RNP nuclear transport 51

Table 4.1 Comparison of nuclear transport pathways by competition 73

Abstract

This thesis presents investigations into aspects of oocyte-type 5S ribosomal RNA (5S rRNA) nuclear transport in *Xenopus laevis* oocytes. Oocyte-type 5S rRNA synthesis begins in the nucleus of early-stage oocytes before other ribosomal RNAs and proteins are available, and the RNA is subsequently stored in the cytoplasm in specific complexes with proteins as 42S and 7S ribonucleoprotein particles (RNPs). 5S rRNA remains sequestered in the cytoplasm until it receives the signal to return to the nucleus to be incorporated into 60S ribosomal subunits; a process occurring in the nucleolus. Movement of 5S rRNA into the nucleus is concurrent with the maximal synthesis of the other ribosomal RNAs and ribosomal proteins at the later stages of oogenesis. At this time, 5S rRNA interacts with ribosomal protein L5 to form pre-ribosomal 5S RNPs. By microinjecting *in vitro*-synthesised, labelled 5S rRNA and 5S RNPs into late-stage *Xenopus* oocytes, I have analysed the mechanism of 5S rRNA localisation to the nucleus. The results presented in this thesis provide evidence for a model for 5S rRNA nuclear import where the exchange of TFIIIA for L5 binding triggers the switch from cytoplasmic storage to its nuclear accumulation. It is likely that L5 provides a nuclear localisation signal which interacts with components of the nuclear transport machinery used by nuclear proteins, enabling the 5S RNP to be translocated into the nucleus through the channel provided by the nuclear pore complex. The process of 5S rRNA nuclear import requires physiological temperatures and metabolic energy, and involves interactions with nuclear pore complex glycoproteins.

Abbreviations

ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
GlcNAc	*N*-Acetylglucosamine
GTP	Guanosine Triphosphate
HIV-1	Human Immunodeficiency Virus Type-1
PBS	Phosphate Buffered Saline
P(lys)-BSA	BSA Conjugated with the SV40 T Antigen NLS
NLS	Nuclear Localisation Signal
NPC	Nuclear Pore Complex
RNP	Ribonucleoprotein Particle
rRNA	Ribosomal Ribonucleic Acid
SDS	Sodium Dodecylsulphate
snRNA/RNP	Small Nuclear Ribonucleic Acid/Ribonucleoprotein Particle
snoRNA/RNP	Small Nucleolar Ribonucleic Acid/Ribonucleoprotein Particle
SV40	Simian Virus 40
TFIIIA	Transcription Factor IIIA
WGA	Wheat Germ Agglutinin

Chapter One
General Introduction

1.1 The Nuclear Membrane as a Selective Barrier

In eukaryotic cells the nuclear membrane forms a barrier between the genetic material in the nucleus and the site of expression of protein-coding genes in the cytoplasm. Transport of molecules and ions in both directions across this membrane is therefore an important process in regulating gene expression during the development and growth of a cell and in response to a changing environment. Proteins such as transcription factors, steroid hormone receptors, and enzymes need to move into the nucleus to interact with DNA during the process of RNA transcription, and histone proteins also have to undertake transport through the nuclear membrane to interact with DNA. RNA also undergoes nucleo-cytoplasmic transport: the RNA-protein complexes involved in processing messenger RNA are assembled in the cytoplasm and function in the nucleus, the processed mRNA must move out to the cytoplasm to be used as a template for protein synthesis, and transfer RNA and signal recognition particle RNA (involved in protein translocation through the endoplasmic reticulum membrane) are exported from the nucleus after synthesis to their site of action in the cytoplasm. Finally, ribosomes, the machinery upon which proteins are manufactured, are assembled from a collection of proteins and RNAs and this involves coordinated movement of ribosomal components between these two cellular compartments.

In spite of the central importance of the process of nuclear transport, the construction of a model for the transport process, integrating the molecular interactions and cellular components involved, has remained enigmatic. Most of the studies to date have focused on the mechanism of protein transport from cytoplasm to nucleus (for recent reviews see: Wagner *et al.*, 1990; Garcia-Bustos *et al.*, 1991; Silver, 1991; Stochaj & Silver, 1992). Proteins for which nuclear transport processes have been characterised include: steroid hormone receptors, such as the progesterone receptor (Guichon-Mantel *et al.*, 1991); growth factors (reviewed in Jans, 1994); histones (Breeuwer & Goldfarb, 1990); ribosomal proteins (Moreland *et al.*, 1985); components of signalling pathways such as calmodulin (Pruschy *et al.*, 1994) and the catalytic

subunit of protein kinase C (Harootunian *et al.*, 1993); and viral proteins (Kemler *et al.*, 1994; Schliephake & Rethwilm, 1994). Initially, it was presumed that the passage of viral proteins and nucleic acids into the nucleus for viral replication would occur exclusively when the nuclear membrane is absent during cell division, as is the case with many oncogenic viruses such as the murine leukaemia virus (Roe *et al.*, 1993; Lewis & Emerman, 1994). However, there is now evidence that transport across the membrane is an important process in the infection of cells with some viruses, including simian virus 40 (SV40; Yamada & Kasamatsu, 1993), influenza virus (Martin & Helenius, 1991a,b) and human immunodeficiency virus type-I (HIV-I; Meyer & Malim, 1994; Stevenson, 1994).

RNAs are most likely to exist within the cell exclusively in specific complexes with proteins as ribonucleoprotein particles (RNPs), which serve to stabilise the molecules and to regulate their function. The nucleo-cytoplasmic trafficking of RNPs has been the focus of much recent research. The mechanism of transport out of the nucleus has been investigated for tRNA (Zasloff *et al.*, 1982; Tobian *et al.*, 1985), pre-mRNA (Eckner *et al.*, 1991; Dargemont & Kühn, 1992; Williams *et al.*, 1994), signal recognition particle RNA (He *et al.*, 1994), uridine-rich small nuclear RNAs (U snRNAs; Hamm & Mattaj, 1990; Terns *et al.*, 1993), 5S rRNA (Guddat *et al.*, 1990), and ribosomal subunits (Bataillé *et al.*, 1990). Some of the U snRNAs also undergo transport across the nuclear membrane in the other direction, and this import process has been studied as well (Fischer & Lührmann, 1990; Hamm *et al.*, 1990; Michaud & Goldfarb, 1991, 1992). The process of nuclear transport must therefore accommodate a variety of molecules and complexes, and operate in both directions across the nuclear membrane.

1.2 Movement of 5S rRNA Between the Nucleus and Cytoplasm

Ribosome biogenesis requires movement across the nuclear membrane of four different RNA molecules (28S, 18S, 5.8S and 5S ribosomal RNAs [rRNAs]) and approximately eighty ribosomal proteins. In somatic cells, ribosomal proteins move from their site of synthesis in the cytoplasm to the nucleus, where they combine with ribosomal RNAs to form pre-ribosomal particles. This process occurs in the

nucleolus, the subnuclear compartment which is the site of ribosome synthesis (Hadjiolov, 1985). The 60S and 40S ribosomal subunits formed are then exported to the cytoplasm to take part in protein synthesis, with 18S rRNA in the 40S subunit and 28S, 5.8S and 5S rRNA in the 60S subunit (Hadjiolov, 1985; Hill *et al.*, 1990). In oocytes of the South African clawed frog, *Xenopus laevis*, the requirement for large numbers of ribosomes (approximately 10^{12} in a fully grown oocyte; Hausen & Riebesell, 1991) for the burst of protein synthesis accompanying embryogenesis, results in additional steps to the process of ribosome production.

In *Xenopus* oocytes there are two families of developmentally regulated 5S rRNA genes: oocyte-type and somatic-type (Wegnez *et al.*, 1972; Ford & Southern, 1973; reviewed in Wolffe & Brown, 1988). Oocyte-type 5S rRNA is transcribed in the developing oocyte from approximately 20 000 copies of the gene per haploid genome, but its transcription is greatly reduced during embryogenesis and is blocked in somatic cells. Somatic-type 5S rRNA differs from oocyte-type at six nucleotides and is transcribed from approximately 400 gene copies per genome throughout the development of the frog, including in oocytes (Wormington & Brown, 1983). These two types of 5S rRNA have been found to follow different pathways in oocytes with respect to interaction with proteins in RNPs and intracellular localisation, with oocyte-type 5S rRNA being specialised for storage, and somatic-type ready for the production of ribosomes in the mature oocyte (Allison *et al.*, 1995).

Oocyte-type 5S rRNA synthesis begins in early previtellogenic oocytes before other ribosomal components are available (Mairy & Denis, 1971). After synthesis in the nucleus, 5S rRNA moves to the cytoplasm for storage in two different RNPs: either in a 1:1 ratio with transcription factor IIIA (TFIIIA) as 7S RNPs (Honda & Roeder, 1980; Pelham & Brown, 1980); or with one of various tRNAs, a protein related to the elongation factor EF-1α, and p43 (a protein related to TFIIIA) as 42S RNPs (Picard *et al.*, 1980; Mattaj *et al.*, 1983; Joho *et al.*, 1990). Although somatic-type 5S rRNA is synthesised in oocytes, it does not appear to be stored long-term (Denis & Wegnez, 1977). Therefore, the six nucleotide difference between oocyte-type and somatic-type 5S rRNAs appears to confer the storage property on oocyte-type when compared with somatic-type, as demonstrated by its preferential binding to TFIIIA *in vivo* (Allison *et al.*, 1995).

At vitellogenesis, the developmental stage encompassing ribosome synthesis, 18S, 5.8S and 28S rRNA synthesis occurs simultaneously from multiple repeats of the rDNA by RNA polymerase I and is concurrent with ribosome assembly in the amplified nucleoli (Mairy & Denis, 1971; Picard & Wegnez, 1979; Picard *et al.*, 1980). When ribosome synthesis is maximal, oocyte-type 5S rRNA is released from storage in the cytoplasm and moves into the nucleus to join other ribosomal components being assembled into pre-ribosomal particles in the nucleolus (Allison *et al.*, 1991, and references therein). After release from 7S and 42S RNPs, 5S rRNA binds with ribosomal protein L5 to form 5S RNPs which are detectable in the cytoplasm (Allison *et al.*, 1991). In ribosomes treated with EDTA, the 5S RNP is released intact (Blobel, 1971), demonstrating that the 5S rRNA/L5 interaction is stable through to its incorporation into functioning ribosomes. Therefore, it is likely that 5S rRNA is in the form of the stable pre-ribosomal 5S RNP as it moves into the nucleus. The final journey of 5S rRNA across the nuclear membrane is out of the nucleus in the form of the 60S ribosomal subunit, to become part of the functioning ribosome in the cytoplasm (reviewed in Ware & Khanna-Gupta, 1990). In somatic cells, the majority of newly synthesised 5S rRNA is not likely to enter the cytoplasm, but forms 5S RNPs with L5 in the nucleus and is targeted directly to the nucleoli (Steitz *et al.*, 1988). However, a cytoplasmic pool of 5S rRNA has been reported in rat liver cells (Ogata *et al.*, 1993), and somatic-type 5S rRNA microinjected into the oocyte cytoplasm is able to enter the nucleus (Allison *et al.*, 1995), suggesting that somatic-type 5S rRNA has the potential to re-enter the nucleus after a cytoplasmic excursion.

In summary, the pathway to ribosome incorporation for *Xenopus* oocyte-type 5S rRNA involves various 5S rRNA-protein interactions and passage across the nuclear membrane in both directions during oocyte development. Therefore, this molecule provides an ideal system for the investigation of the process of RNA nucleo-cytoplasmic transport and RNA-protein interactions involved. The pathway of 5S rRNA from storage in the cytoplasm to the nucleus for ribosome assembly is the focus of this thesis, with an investigation of the mechanism by which 5S rRNA is targeted to the nucleus. This chapter provides a review of the process of nuclear transport, which will be related to 5S rRNA transport throughout the rest of the thesis.

1.3 The Structure and Function of the Nuclear Pore Complex

1.3.1 Basic structure and function

The transport of molecules and ions in both directions across the nuclear membrane occurs exclusively through aqueous channels formed by nuclear pore complexes (NPCs; for recent reviews see: Akey, 1992; Forbes, 1992; Hurt, 1993; Panté & Aebi, 1993, 1994; Agutter & Prochnow, 1994; Hinshaw, 1994). NPCs are assemblies of multiple protein subunits that span the two layers of the nuclear envelope, forming a junction between the inner and outer membranes. These structures are composed of two rings, facing the cytoplasm and nucleoplasm respectively, between which is sandwiched a central spoke-like assembly containing the transporter through which nucleocytoplasmic trafficking takes place (Akey, 1989; 1990; Hinshaw *et al.*, 1992). The complex has a large central channel, through which macromolecules up to a diameter of 26 nm can be transported in a facilitated manner (Feldherr *et al.*, 1984). This central channel has been proposed to be in the form of an iris-like assembly which expands to provide a larger functional diameter and allows passage of macromolecules in an energy- and signal-dependent manner (Akey, 1990). The large NPC channel is surrounded by smaller peripheral channels that may serve to allow the passive diffusion of ions and molecules of less than 9 nm in diameter (Hinshaw *et al.*, 1992). However, recent evidence suggests that movement of some ions through the nuclear pore complex may occur via a regulated process, and not by passive diffusion (Bustamante, 1992, 1994; Himpens *et al.*, 1994a,b; Mazzanti *et al.*, 1994).

Facilitated transport of proteins through the NPC has been separated into two steps (Newmeyer & Forbes, 1988; Richardson *et al.*, 1988; Akey & Goldfarb, 1989). In the first step the protein binds at the nuclear pore complex in a mechanism which depends on a specific signal provided by the protein, and does not require metabolic energy. The second step requires metabolic energy and involves the translocation of the protein through the nuclear pore channel. These two steps of import have been shown to be dependent on factors present in the cytosol that must interact with both the molecule or particle being transported and the NPC (for recent reviews see:

Powers & Forbes, 1994; Simos & Hurt, 1995). The role of these factors in nuclear transport will be discussed further in Section 1.5.

Only approximately 15% of nuclear pore complex proteins have been identified (Starr & Hanover, 1992; Panté & Aebi, 1994). Some of these proteins have a role in the maintenance of NPC structure, while others are presumably involved in the regulation of facilitated nucleo-cytoplasmic transport. In the next section, the potential roles for some of the identified NPC proteins are discussed.

1.3.2 Specific proteins of the NPC

The majority of vertebrate NPC proteins examined to date are members of a family of glycoproteins that possess *N*-acetylglucosamine (GlcNAc) residues joined by O-glycosyl linkages to serine and threonine residues on the polypeptides (Davis & Blobel, 1987; Hanover *et al.*, 1987; Starr & Hanover, 1990). These proteins, collectively termed nucleoporins, occupy peripheral sites on the NPC at both the cytoplasmic and nucleoplasmic faces, as determined by immunofluorescence and immunoelectron microscopy of nuclear envelopes with antibodies raised against nuclear membrane extracts (Davis & Blobel, 1986; Park *et al.*, 1987; Snow *et al.*, 1987). Some of these GlcNAc-bearing pore proteins possess a highly conserved repeated peptide motif of FXFG or GLFG (single letter code for amino acids, where X codes for any amino acid), which appears to be important for interaction with soluble components of the nuclear transport machinery (for reviews see: Fabre & Hurt, 1994; Rout & Wente, 1995).

The essential role of peripheral nucleoporins in the functioning of the NPC in nucleo-cytoplasmic transport has been demonstrated by the construction of NPCs specifically lacking the GlcNAc-bearing proteins (Finlay & Forbes, 1990; Finlay *et al.*, 1991). These NPCs retained their structural integrity and diffusional channel, but were found to be defective for nuclear protein transport. Monoclonal antibodies raised against nucleoporins and microinjected into *Xenopus* oocytes have been found to inhibit the facilitated nucleocytoplasmic transport of proteins and RNA, but do not affect passive diffusion through the NPC (Snow *et al.*, 1987; Featherstone *et al.*, 1988). Recent studies have demonstrated that nucleoporins in this group interact with

factors involved in both docking of nuclear proteins at the nuclear pore complex (Kraemer *et al.*, 1995; Moroianu *et al.*, 1995a,b; Paschal & Gerace, 1995; Radu *et al.*, 1995a,b), and in subsequent translocation through the nuclear pore complex (Dingwall *et al.*, 1995; Hartmann & Görlich, 1995; Yokoyama *et al.*, 1995). One of the GlcNAc-bearing nucleoporins, p62, is symmetrically localised on both the cytoplasmic and nucleoplasmic sides of the NPC (Cordes *et al.*, 1991), indicating that it could have a role both in nuclear import and export. This is corroborated by evidence of an interaction between p62 and mRNA which is being exported from the nucleus (Dargemont *et al.*, 1995).

Filamentous elements have been observed extending from both cytoplasmic and nuclear faces of the NPC (for review see Davis, 1995). These filaments give an asymmetric aspect to NPC structure, with short fibrils perpendicular to the nuclear membrane at the cytoplasmic face, and a complex fish net-like structure emanating from the nuclear side (Jarnaik & Aebi, 1991; Ris, 1991; Goldberg & Allen, 1992; 1993). Nucleoporins have been isolated that may be constituents of these fibres; they include Nup358 (Wu *et al.*, 1995a) and Nup214 (Kraemer *et al.*, 1994) at the cytoplasmic side, and Nup153 (Sukegawa & Blobel, 1993) and Nup98 (Radu *et al.*, 1995b) facing the nucleoplasm. Interaction of the cytoplasmically disposed nucleoporins with factors involved in the docking stage of nuclear protein import suggest that the fibres are involved in the recognition of the transported protein at the NPC (Moroianu *et al.*, 1995b; Radu *et al.*, 1995b). The NPC fibres may also form a part of a complex series of tracks that link the cytoplasm and nucleus through the cytoskeleton and karyoskeleton, and which may provide the physical pathways for nucleo-cytoplasmic transport. Messenger RNA molecules have been observed attached to filamentous tracks in the nucleus (Huang *et al.*, 1994), and fibrils can be seen linking NPCs with nucleoli, providing possible paths for the nuclear transport of ribosomal components (Cordes *et al.*, 1993). Indeed, a nucleolar protein, Nopp140, has been visualised during transport attached to filamentous tracks emanating from the nucleolus (Meier & Blobel, 1992).

Information on the biochemical make-up of the NPC is far from complete; however, functional studies using pores depleted of specific proteins as well as genetic studies in yeast, are yielding promising results in the determination of the function of NPC proteins (for review see: Doye & Hurt, 1995).

1.4 Nuclear Transport Signals

1.4.1 Proteins

Proteins which function in a cellular compartment other than the site of protein synthesis in the cytoplasm require a specific signal for appropriate targeting after (or during the process of) translation. Thus, the targeting of proteins to the endoplasmic reticulum and mitochondria, requires specific signals within the amino acid sequence of the molecules (Walter & Lingappa, 1986). Facilitated nuclear transport of karyophilic proteins also requires specific signals called nuclear localisation signals (NLSs; for reviews see: Roberts, 1989; Silver & Goodson, 1989; Wagner *et al.*, 1990; Silver, 1991; Garcia-Bustos *et al.*, 1991a; Richter & Standiford, 1992; Stochaj & Silver, 1992). The NLS appears to be involved in the energy-independent binding step of transport through the NPC (Akey & Goldfarb, 1989; Newmeyer & Forbes, 1988). NLSs have been characterised for yeast (Moreland *et al.*, 1985; Nelson & Silver, 1989), mammalian (Shaulsky *et al.*, 1990), amphibian (Robbins *et al.*, 1991; Standiford & Richter, 1992), viral (Kalderon *et al.*, 1984; Yeh *et al.*, 1990) and plant (Varagona *et al.*, 1992) proteins. The mere presence of an NLS on a molecule is sufficient to enable the NPC channel to open and allow its passage, even to the extent that 20-260 Å gold particles coated with NLS-containing proteins are readily transported through the NPC (Dworetzky & Feldherr, 1988).

NLSs have been investigated using three major techniques: deletion analysis, whereby portions of the amino acid sequence are deleted to assess their importance with regard to nuclear transport (Dingwall *et al.*, 1982); mutational analysis resulting in amino acid substitution (Robbins *et al.*, 1991; Standiford & Richter, 1992); and by gene fusion of the putative NLS to a non-nuclear protein (Moreland *et al.*, 1985; Lanford *et al.*, 1990; Yeh *et al.*, 1990). A functional NLS is defined as the minimal

amino acid sequence both necessary and sufficient to induce nuclear localisation of a non-nuclear protein to which it is fused. Unlike the signals targeting proteins to the endoplasmic reticulum and mitochondria, NLSs are not proteolytically removed during the transport process.

No consensus amino acid sequence for nuclear localisation has been determined, but there are common features between those identified to date. NLSs are typically short sequences (approximately 8 to 10 amino acids) that contain a high proportion of the positively charged amino acids lysine and arginine, with the frequent presence of a flanking proline or glycine residue. The most studied NLS is that of the SV40 large T antigen, which has a NLS composed of the short stretch of basic amino acids: PKKKRKV (Kalderon *et al.*, 1984; Lanford *et al.*, 1984). Subsequently, more complex types of NLS have been identified that involve more than one sequence of amino acids. Nucleoplasmin, an abundant nuclear protein in *Xenopus*, has an NLS composed of two basic sequences separated by a spacer region of 10 amino acids (Dingwall *et al.*, 1988; Robbins *et al.*, 1991). Similar NLSs have been described for the *Xenopus* N1 protein (Kleinschmidt & Seiter, 1988) and the nucleolar protein No38 (Schmidt-Zachmann *et al.*, 1987). Other types of NLS are composed of several clusters of basic amino acids, as has been found for the progesterone receptor (Ylikomi *et al.*, 1992) and the oncogene product p53 (Shaulsky *et al.*, 1990). Not all protein NLSs that have been identified conform to the model involving short sequences of basic amino acids. In some proteins the amino acids that make up the NLS are dispersed throughout a large segment of the protein, such as the U1 snRNP-specific U1A protein (Kambach & Mattaj, 1992), the U2 snRNP-specific U2B″ protein (Kambach & Mattaj, 1994) and *Saccharomyces cerevisiae* Gal4 protein (Silver *et al.*, 1988). These residues may form a more compact NLS when the protein is in its folded conformation. Furthermore, the nuclear localisation of the pre-mRNA/mRNA binding protein hnRNP A1 protein has been found to be dependent on a novel glycine-rich domain at its carboxyl terminus (Siomi & Dreyfuss, 1995). A summary of types of protein NLS is provided in Table 1.1.

The positioning of the NLS in a protein is important for its function, presumably due to the necessity of exposing the signal for interaction with NLS binding proteins or receptors involved in nuclear transport. Nelson and Silver (1989) determined that

Table 1.1 Types of protein nuclear localisation signal

Nuclear localisation signal	Example protein(s)	Reference
sequence of basic amino acids PKKKRKV	SV40 T antigen	Kalderon *et al.*, 1984 Lanford *et al.*, 1986
two sequences of basic amino acids separated by a spacer region	*Xenopus* nucleoplasmin *Xenopus* N1 protein *Xenopus* NO38	Dingwall *et al.*, 1988 Robbins *et al.*, 1991 Kleinschmidt & Seiter, 1988 Schmidt-Zachmann *et al.*, 1987
clusters of basic amino acids	rabbit progesterone receptor human, mouse, rat and *Xenopus* p53	Ylikomi *et al.*, 1992 Shaulsky *et al.*, 1990
dispersed over a large region of basic amino acids	*Xenopus* U1A protein *Xenopus* U2B″ protein yeast Gal4	Kambach & Mattaj, 1992 Kambach & Mattaj, 1994 Silver *et al.*, 1988
glycine-rich domain at C-terminus	human hnRNP A1 protein	Siomi & Dreyfuss, 1995

the ability of the SV40 T antigen NLS to function in nuclear transport partly depended on the (non-nuclear) protein to which it had been experimentally fused, while Roberts *et al.* (1987) found that the positioning of a NLS within the protein sequence of the non-nuclear protein pyruvate kinase affected its nuclear targeting ability. In addition, the efficiency of nuclear targeting of a NLS is greatly enhanced if multiple copies of the signal are present (Roberts *et al.*, 1987; Dworetzky *et al.*, 1988). Sequences flanking the NLS may also be important for the regulation of NLS function. The phosphorylation of sites adjacent to an NLS has been found to increase the efficiency of nuclear accumulation (Rihs & Peters, 1989; Rihs *et al.*, 1991; Moll *et al.*, 1991; Jans & Jans, 1994). Furthermore, cyclic AMP-dependent protein kinase has been implicated in the control of nuclear localisation (Gauthier-Rouvière *et al.*, 1995). Regions adjacent to the NLS could potentially provide a mechanism for the control of sub-cellular localisation of a protein, perhaps during development. An example of a

developmentally regulated NLS, which functions in *Xenopus* oocytes but not in *Xenopus* somatic cells, has been found in the oncoprotein encoded by the adenovirus 5 E1a gene (Standiford & Richter, 1992). It is possible that this mechanism usurped by the viral protein is operating for those *Xenopus* proteins that undergo a shift in nucleo-cytoplasmic distribution during embryogenesis (Dreyer, 1989).

1.4.2 RNAs/RNPs

In addition to NLSs for proteins that are transported across the nuclear membrane, there must also be signals that target RNAs into and out of the nucleus. All RNA molecules are likely to exist in specific associations with proteins as RNPs during functioning and transportation within the cell. Direct evidence for this comes from electron microscopic studies which have visualised export through the NPC of pre-mRNA bound with specific proteins (Mehlin *et al.*, 1992; 1995). The nuclear import and export signals on transported RNPs could therefore reside either in the amino acid sequence of the protein or proteins, in the nucleotide sequence of the RNA, or in a combination of both. In fact, examples of all these scenarios have been identified for different RNPs, and the requirements for nuclear transport of RNPs appear to be both complex and varied (Fischer & Lührmann, 1990; Hamm & Mattaj, 1990; Fischer *et al.*, 1991; 1993; 1994a,b; 1995; Michaud & Goldfarb, 1992; Terns *et al.*, 1993; Jarmolowski *et al.*, 1994; reviewed in Izauralde & Mattaj, 1995).

Studies on the requirements for RNA nuclear transport were first undertaken for the nuclear export of tRNA (Zasloff *et al.*, 1982). The process of nuclear export of this small molecule was found to be carrier-mediated, and mutational analysis revealed that export required many regions of the nucleotide sequence (Tobian *et al.*, 1985). The mutations that had an effect on export were found to result in changes to stem-loop structures on the tRNA molecule, indicating that maintenance of secondary structure is important for the interactions involved in nuclear transport. It is unknown whether these nucleotide sequences interact directly with the nuclear transport machinery, or provide a binding site (or sites) for a protein which is transported complexed with the RNA and acts to target the tRNA for export. Such a protein could be glyceraldehyde-3-phosphate dehydrogenase, whose binding has been related to tRNA export (Singh & Green, 1993).

The nuclear export of mRNA is also a process that requires *cis*-acting signals within the RNA structure. The m7GpppN (monomethylated) cap structure, a modified guanosine nucleotide which is added to the 5′ end of the RNA during transcription by RNA polymerase II, is involved in signalling nuclear export of the molecule (Hamm & Mattaj, 1990; Dargemont & Kühn, 1992; Jarmolowski *et al.*, 1994). The possession of an altered cap structure results in reduced nuclear export capability for mRNA (Hamm & Mattaj, 1990), and the presence of an analogue of the cap inhibits the export of mRNA microinjected into oocytes (Dargemont & Kühn, 1992). Other elements of the mRNA structure also appear to be involved in signalling export. The 3′ end of histone mRNA (Eckner *et al.*, 1991), specifically the 3′ stem-loop region of this molecule (Williams *et al.*, 1994), has been implicated in its export to the cytoplasm.

The assembly of U snRNPs, which function in the processing of mRNAs, involves transport across the nuclear membrane in both directions and is therefore an interesting process in terms of RNA nuclear transport signals (reviewed by: Izaurralde & Mattaj, 1992). The U snRNAs are transcribed in the nucleus and move into the cytoplasm where they form specific associations with proteins. Two different types of proteins are involved in these interactions: those common to all particles (the Sm proteins), and those specific to the individual U snRNP (i.e., U1, U2, U4, and U5 snRNPs; Parry *et al.*, 1989). The assembled RNPs then move back into the nucleus to perform their splicing function (reviewed by Lührmann *et al.*, 1990). U snRNAs therefore presumably require a signal for nuclear export, and the U snRNPs require a signal for nuclear import. Hamm and Mattaj (1990) determined that, as with mRNA, the likely signal that targets U1 snRNA from the nucleus is the monomethylguanosine cap. U1 snRNA possessing a different capping nucleotide, produced experimentally by transcription with RNA polymerase III, was shown to be retained in the nucleus. A more recent study has demonstrated that the monomethylated cap alone does not act as the signal for nuclear export of U1 snRNA; there is also a requirement for the 3′ stem-loop structure (Neuman de Vegvar & Dahlberg, 1990; Terns *et al.*, 1993). The study of Terns *et al.* (1993) also showed that efficient nuclear export of U1 snRNA after synthesis involves sequences within the 5′ terminal 124 nucleotides.

After export to the cytoplasm, the monomethylguanosine structure is converted to a trimethylguanosine cap (m_3G; Lührmann *et al.*, 1990). This trimethylguanosine cap nucleotide is an essential requirement for U1 snRNA re-import into the nucleus in *Xenopus* oocytes, as cytoplasmically microinjected uncapped U1 snRNA, or U1 snRNA capped with the 'unnatural' ApppG cap, remained cytoplasmic (Fischer & Lührmann, 1990). By constructing mutants of U1 snRNA, Hamm *et al.* (1990) were able to determine that the binding site for Sm proteins on the RNA molecule, in addition to the trimethylguanosine cap, are essential for nuclear import. Thus, the signal that targets U1 snRNPs for nuclear import is a combination of both the RNA structure and a component of the bound protein. U2 snRNP import to the nucleus also appears to have a requirement for the trimethylguanosine cap, since U2 snRNA synthesised without this capping nucleotide and microinjected into the oocyte cytoplasm, is excluded from the nucleus (Fischer *et al.*, 1991). The presence of an excess of a 2,2,7mGpppG dinucleotide analogue of the cap structure also inhibits the nuclear import of U1 and U2 snRNAs, presumably by competing for a factor to which this import signal binds (Fischer *et al.*, 1991; Michaud & Goldfarb, 1992). However, the nuclear import of U4 and U5 snRNPs has a less stringent requirement for the trimethylguanosine cap (Fischer *et al.*, 1991).

In somatic cells from mouse, monkey and *Xenopus*, it has been found that, although the trimethylguanosine cap accelerates the nuclear import of U1 snRNPs, it is not an essential requirement for nuclear targeting, with the Sm domain alone potentially providing sufficient signal (Fischer *et al.*, 1994b). An earlier study also demonstrated that the Sm core domain contains a transport signal that is independent of the trimethylguanosine cap (Fischer *et al.*, 1993). The U1 snRNP may therefore be an example of a developmentally regulated NLS, with different factors involved in the import process in *Xenopus* oocytes and in somatic cells (Marshallsay & Lührmann, 1994).

Finally, in *Xenopus* oocytes the nuclear export of 5S rRNA, the molecule which is the focus of this thesis, is dependent on binding to either the protein TFIIIA (as 7S RNPs) or ribosomal protein L5 (as 5S RNPs), since 5S rRNA mutants that are incapable of binding these proteins are retained in the oocyte nucleus after synthesis (Guddat *et al.*, 1991). It is therefore possible that the NLS targeting the export of 5S

rRNA resides on one of these proteins. Although there is evidence of a requirement for specific protein associations during export, the proteins involved in 5S rRNA nuclear import have not been investigated. Therefore, the role of ribosomal protein L5 in the nuclear import of 5S rRNA is examined in this thesis.

1.5 Nuclear Localisation Signal Receptors and Other *Trans*-Acting Factors in Nuclear Transport

1.5.1 Saturability of nuclear transport

The presence of NLSs on karyophilic molecules implies the existence of receptors to which these signals bind in the pathways for nuclear transport. Investigation of factors involved in recognition and targeting of karyophilic proteins for nuclear import has led to the discovery of proteins in the cytosol, as well as those associated with the NPC, that are essential to both the binding and translocation steps of transport through the pore channel (reviewed in Powers & Forbes, 1994). However, it is likely that a large number of nuclear transport factors remain to be discovered, which could act specifically in the import and export of specific classes of proteins and RNPs.

Indirect evidence for the involvement of specific binding factors, or receptors, in nuclear transport has been provided by studies showing that nuclear transport is a saturable process. For example, the rate of nuclear import of a radio-labelled synthetic karyophilic protein, P(lys)-BSA (bovine serum albumin [BSA] conjugated with multiple copies of the minimal SV40 T antigen NLS), is reduced in the presence of 'excess' unlabelled P(lys)-BSA (Michaud & Goldfarb, 1991). Likewise, the nuclear export of tRNA (Zasloff *et al*, 1983) and ribosomal subunits (Bataillé *et al.*, 1990) has been shown to involve a cellular component (or components) present in limiting amounts. Further studies have been able to demonstrate the utilisation of common components of the nuclear transport machinery between different karyophiles (nuclear molecules or complexes), by testing import of tagged molecules in the presence of another karyophile 'in excess' (Michaud & Goldfarb, 1991,1992; Dargemont & Kühn, 1992; Jarmolowski *et al.*, 1994). In this way, karyophiles can be grouped into

different classes, depending on their requirements for a limiting factor, which may prove to be an NLS receptor. It is conceivable that these groupings of molecules to be transported may enable the co-ordination and regulation of nuclear transport on the basis of the availability of NLS receptors at any given time during the growth and development of a cell, and in response to a changing environment.

Biochemical approaches have subsequently been used in the identification of factors essential for NLS recognition and nuclear transport. Firstly, NLS-binding proteins have been isolated on the basis of peptide cross-linking, affinity chromatography, or ligand blotting using identified NLSs. Proteins able to bind the SV40 T antigen NLS have been identified in rat liver cells (Adam *et al.*, 1989; Benditt *et al.*, 1989), yeast (Lee & Mélèse, 1989), human tissue culture cells (Li & Thomas, 1989), and plants (Hicks *et al.*, 1995; Hicks & Raikhel, 1995). It was initially assumed that NLS-binding proteins would reside predominantly at the nuclear envelope, however the NLS-binding proteins identified were found to be localised in the nucleoplasm and cytoplasm, as well as at the nuclear envelope (Benditt *et al.*, 1989), and therefore could not be classed exclusively as components of the NPC. Furthermore, these studies have been unable to determine whether the isolated NLS-binding proteins have an actual role in the process of nuclear transport.

1.5.2 Characterisation of factors involved in the two steps of nuclear protein import

Recently, new biochemical techniques have been used to isolate specific factors associated with steps in nuclear protein import. These involve the use of cell-free systems, which enable the testing of isolated fractions of cytosol for the presence (or absence) of a factor required for nuclear transport. One such system utilises *Xenopus* egg extracts in the formation of transport-competent nuclei isolated from rat liver, or constructed *de novo* around added DNA (Newmeyer & Forbes, 1990; Newmeyer *et al.*, 1986b). Another system involves the use of cultured mammalian cells which have been permeabilised by treatment with digitonin, which selectively perforates the plasma membrane while leaving the nuclear membrane intact and transport-competent (Adam *et al.*, 1990). Using either of these systems, the nuclear import of proteins has been shown to be dependent on factors present in the cytosol, with the binding and

translocation steps of nuclear import dependent on components of two different cytosolic fractions which contain specific transport factors (Adam *et al.*, 1990; Newmeyer & Forbes, 1990; Moore & Blobel, 1992).

1.5.2.1 NLS binding and docking at the NPC

One fraction of cytosol used in the *in vitro* transport systems, termed fraction A (Moore & Blobel, 1992), or NIF1 (Newmeyer & Forbes, 1990), was found to be required for the docking of a karyophilic protein at the NPC, but was not sufficient for translocation through the NPC channel. The factors responsible for this activity have subsequently been isolated and have been found to be highly conserved between species (as described below, and reviewed by: Simos & Hurt, 1995). In addition, it has been determined that there are separate factors involved in NLS binding and docking at the nuclear pore complex, resulting in a further division of the 'first' step of nuclear protein import into interaction between the NLS and its receptor, followed by association of the complex with proteins of the nuclear pore.

The first direct evidence for the involvement of NLS-binding proteins in nuclear transport came from the study of Adam and Gerace (1991). They isolated two polypeptides from bovine erythrocytes, based on their ability to bind the SV40 T antigen NLS, which were able to stimulate nuclear transport *in vitro*. Proteins analogous to this heterodimeric 54/56 kD nuclear transport receptor have subsequently been isolated from *Xenopus* eggs (importin-α, or importin-60, Görlich *et al.*, 1994, 1995a,b), rat liver (karyopherin-α, Radu *et al.*, 1995a), human cultured cells (karyopherin-α, or Rch1, Moroianu *et al.*, 1995a,b; hSRP1α, Weis *et al.*, 1995), mouse cultured cells (m-importin, Imamoto *et al.*, 1995b), and yeast (karyopherin-α or Kap60p, Enenkel *et al.*, 1995). In addition, a protein isolated from goat uterus, p55, appears to act as the NLS-binding protein for the estrogen receptor (Nirmala & Thampan, 1995 a,b). Importin-α (Görlich *et al.*, 1995b) and hSRP1α (Weis *et al.*, 1995) have been found to bind bipartite NLSs as well as the simple type of the SV40 T antigen, suggesting that these receptors may recognise a variety of transport signals.

Although these receptors bind NLSs prior to nuclear import, they do not appear to act alone to target karyophilic proteins to the nuclear pore, but require at least one

other factor. A factor with this associated activity, p97, was first isolated from bovine erythrocytes (Adam & Adam, 1994; Chi *et al.*, 1995). Other proteins required for docking at the nuclear pore have been isolated from other species, and found to directly interact with nuclear pore complex glycoproteins (Kraemer *et al.*, 1995; Moroianu *et al.*, 1995a,b; Paschal & Gerace, 1995; Radu *et al.*, 1995a,b). These proteins are *Xenopus* importin-β (or importin 90; Görlich *et al.*, 1995a,b), rat karyopherin-β (Radu *et al.*, 1995a,b), human karyopherin-β (Moroianu *et al.*, 1995a,b), and yeast karyopherin-β, or Kap95p (Enenkel *et al.*, 1995). It appears that the proteins responsible for NLS binding and subsequent docking at the NPC form a complex after binding of the karyophile, termed the nuclear pore targeting complex by one group (Imamoto *et al.*, 1995a,c). This complex includes the importin α/β (or similar) heterodimer, the protein being imported, and possibly other, as yet unidentified, factors. In the first stage of nuclear targeting, the protein binds the NLS receptor (importin-α type), after which the docking protein binds (importin-β type) and the complex associates with the nuclear pore (Görlich *et al.*, 1995b, Imamoto *et al.*, 1995c). The complex then appears to be translocated through the NPC channel (with the aid of factors described below), and importin-α bound with the transported protein dissociates from importin-β as it enters the nucleoplasm (Görlich *et al.*, 1995b).

1.5.2.2 Translocation

The second step in nuclear protein import, translocation through the nuclear pore complex channel, has been found to be dependent on a second cytosolic fraction (Newmeyer & Forbes, 1990; Moore & Blobel, 1992). A factor responsible for this activity was isolated from *Xenopus* oocytes and was found to be a single protein of 25 kD, identified as the GTP-binding protein Ran, a protein with a sequence related to the Ras family (Moore & Blobel, 1993; 1994a). Ran (**Ra**s-related **n**uclear protein) is an abundant nuclear protein that has been implicated in many nuclear functions via its activity as a GTPase in combination with the guanine nucleotide exchange factor RCC1, and potentially other Ran binding proteins (reviewed in Dasso, 1993; Goldfarb, 1994). The putative active GTP-bound form of Ran is essential for the nuclear import and export of proteins in digitonin permeabilised cells (Melchior *et al.*, 1993; Moroianu & Blobel, 1995). Nuclear protein import in yeast is dependent on GTP

hydrolysis, and the Ran activating protein Rna1p (Corbett *et al.*, 1995; Schlenstedt *et al.*, 1995).

The translocation activity of nuclear protein import is dependent on the presence of another factor, which acts in conjunction with Ran. This protein, p10 (also called pp15 and Ran interacting protein [Ranip]), has been identified in *Xenopus* (Moore & Blobel, 1994b) and has a human homologue, NTF2 (Paschal & Gerace, 1995), although it is not clear as to the role of this protein. Both Ran (Dingwall *et al.*, 1995; Hartmann & Görlich, 1995; Yokoyama *et al.*, 1995) and Ranip (Paschal & Gerace, 1995) have been found to interact directly with nucleoporins, indicating that they may be bound at the NPC during their role in translocation.

The final factor found to date to be associated with protein import is the heat shock protein hsp70, and its cytosolic counterpart hsc70 (Shi & Thomas, 1992; Imamoto *et al.*, 1992; Okuno *et al.*, 1993; Yang & DeFranco, 1994). Hsc70 is co-localised with karyophilic proteins during their transport (Okuno *et al.*, 1993), and antibodies against hsc70 inhibit the nuclear import of karyophilic proteins (Imamoto *et al.*, 1992). The role of hsc70 in nuclear transport is unclear, but there is evidence of an affinity for NLSs (Imamoto *et al.*, 1992). In addition, hsc70 has been found to shuttle between cytoplasmic and nuclear compartments, potentially carrying karyophilic cargoes with it (Mandell & Feldherr, 1990). Hsc70 is also able to associate with the guanine nucleotide exchange factor RCC1, in a complex of proteins that includes Ran (Saitoh & Dasso, 1995), providing a potential link between hsp70/hsc70 and the Ran-mediated step of protein transport.

As summarised in Table 1.2, there have been at least five different soluble factors isolated that are required for nuclear protein import: importin-α/karyopherin-α/NLS-receptor, importin-β/karyopherin-β/97 kD accessory protein, Ran/TC4, Ranip, and hsp/hsc70. These proteins form part of the sequence of events starting with the NLS and resulting in nuclear translocation of the NLS-bearing molecule. It is likely that the requirements for some of these factors vary between different karyophiles, and this may contribute to the regulation of the transport process. Indeed, the requirements for hsp70 have been found to differ between the glucocorticoid receptors from two different cell types (Yang & DeFranco, 1994).

Table 1.2 Factors involved in nuclear protein import

Import step	Factor	Reference
Binding to NLS	bovine 54/56 kD receptor	Adam & Gerace, 1991
	Xenopus importin-α (60 kD)	Görlich *et al.*, 1994; 1995a,b
	mouse importin	Imamoto *et al.*, 1995b
	rat karyopherin-α	Radu *et al.*, 1995a,b
	human SRP1α	Weis *et al.*, 1995
	human karyopherin-α (Rch1)	Moroianu *et al.*, 1995a,b; Görlich *et al.*, 1995a
	yeast karyopherin-α (Srp1p, Kap60p)	Enenkel *et al.*, 1995
Docking at the nuclear pore	bovine 97 kD 'accessory' protein	Adam & Adam, 1994; Chi *et al.*, 1995
	Xenopus importin-β (90 kD)	Görlich *et al.*, 1994; 1995a,b
	rat karyopherin-β	Radu *et al.*, 1995a,b
	human karyopherin-β	Moroianu *et al.*, 1995a,b
	yeast karyopherin-β (Kap95p)	Enenkel *et al.*, 1995
Translocation	*Xenopus* Ran	Moore & Blobel, 1993; 1994a
	Xenopus Ran interacting protein (p10, pp15)	Moore & Blobel, 1994b
	human NTF2 (Ran interacting protein)	Paschal & Gerace, 1995
Unknown	human hsp70/hsc70	Shi & Thomas, 1992; Okuno *et al.*, 1993; Yang & DeFranco, 1994
	rat hsc70	Imamoto *et al.*, 1992

1.5.3 *Trans*-acting factors in RNP nuclear transport

To date, the studies on factors associated with nucleo-cytoplasmic trafficking have largely focused on the import of proteins into the nucleus. Are there specific factors associated with the nucleo-cytoplasmic trafficking of RNPs? Transport competition studies have demonstrated that there are separate pathways for the transport of karyophilic proteins and some of the U snRNAs (Fischer *et al.*, 1991; Michaud & Goldfarb, 1991, 1992; van Zee *et al.*, 1993), and it is likely that different factors are involved in mediating the transport of molecules with such differing

nuclear targeting signals. The nuclear import of the various U snRNPs has been found to occur by three distinct targeting pathways (Michaud & Goldfarb, 1992), and the nuclear export of RNAs is mediated by class-specific components (Jarmolowski *et al.*, 1994), thus indicating the presence of many factors governing these processes. Comparisons of various classes of karyophiles, utilising competition studies, will be further discussed in Chapter Four.

Nuclear import of U1 and U2 snRNPs in digitonin-permeabilised cells is dependent on the addition of an exogenous cytosolic extract, demonstrating the presence of factors in the cytoplasm that promote snRNP transport (Marshallsay & Lührmann, 1994). As reported for the cytosolic fraction involved in the NLS-binding and targeting component of the protein import pathway (Adam *et al.*, 1990; Newmeyer & Forbes, 1990; Adam & Gerace, 1991; Moore & Blobel, 1992), the nuclear transport activity of the cytosol was sensitive to the sulphydryl alkylating agent *N*-ethylmalemide. Furthermore, these factors were found to mediate the differential requirement for the m_3G-cap in oocytes and somatic cells, as the addition of a cytosolic extract from *Xenopus* oocytes conferred cap-dependence on the nuclear transport process, whereas factors from somatic cell cytosol resulted in cap-independent nuclear import (Marshallsay & Lührmann, 1994). This provides evidence for the involvement of cytosolic factors in the developmental regulation of nucleo-cytoplasmic trafficking.

Ran/TC4 has also been implicated in RNA nuclear export through studies of the associated protein RCC1 (Moore & Blobel, 1994a). Nuclear export of mRNA has been found to be defective in mammalian cells expressing a mutant form of the mammalian RCC1, and its yeast homologue PRP20/SRM1/MTR1 (Amberg *et al.*, 1993; Dasso, 1993; Kadowaki *et al.*, 1993), while U3 small nucleolar RNA (snoRNA) intranuclear transport and U1 snRNA export is inhibited in mammalian cultured cells depleted of RCC1 (Cheng *et al.*, 1995). Furthermore, the nuclear export of pre-mRNA is blocked in yeast cells expressing Ran that binds, but does not hydrolyse GTP (Schlenstedt *et al.*, 1995). It is therefore likely that the nucleo-cytoplasmic trafficking of proteins and RNAs/RNPs utilise common components within the cytoplasm and nucleoplasm.

1.6 Shuttling Proteins

Although most proteins and RNPs appear to move across the nuclear membrane unidirectionally, some proteins apparently shuttle constitutively across the nuclear membrane in both directions, potentially ferrying protein or RNP cargoes (reviewed in Laskey & Dingwall, 1993). Such shuttling proteins include: two hsp70-related *Xenopus* proteins (Mandell & Feldherr, 1990); the nucleolar proteins No38, nucleolin (Borer *et al.*, 1989) and Nopp140 (Meier & Blobel, 1992); the yeast RNA-binding protein Npl3 (Flach *et al.*, 1994); the progesterone receptor (Guiochon-Mantel *et al.*, 1991); the hnRNA packaging protein A1 (Piñol-Roma & Dreyfuss, 1992); the HIV-I Rev protein (Meyer & Malim, 1994), and the U1 snRNP-specific protein U1A (Kambach & Mattaj, 1992). These studies have mostly utilised inter-species heterokaryons, whereby cells of two different species are fused creating a cell with two morphologically distinct nuclei. The protein under study is only expressed in one cell type due to the transfection of the expression vector. Therefore, after fusion of the cells the movement of the protein into the nucleus of the other cell type can be observed using fluorescently-labelled antibodies.

Many of the identified shuttling proteins bind RNA, and this suggests that they may play an important role in RNA transport (Piñol-Roma & Dreyfuss, 1992; Flach *et al.*, 1994). In the case of the U1A protein, its shuttling is dependent on the distribution of U snRNA, to which it binds (Kambach & Mattaj, 1992). In addition, the nucleolar shuttling proteins may have a role in the nucleo-cytoplasmic transport of ribosomal components (Borer *et al.*, 1989; Meier & Blobel, 1992). The amino terminus of nucleolin specifically recognises SV40 T antigen-type NLSs (Xue *et al.*, 1993), the type of signal likely to target ribosomal proteins to the nucleus (Moreland *et al.*, 1985; Underwood & Fried, 1990; Schaap *et al.*, 1991). No38 is also capable of binding the SV40 T antigen and HIV-1 Rev NLSs but not the bipartite nucleoplasmin NLS, suggesting that the NLS receptors on these shuttling proteins may be capable of discriminating between different classes of NLS (Szebeni *et al.*, 1995).

An interesting feature of shuttling proteins is the signals that specify import and export, and their ability to be regulated depending on the binding of the karyophile. There is some debate as to whether the transport of shuttling proteins is a facilitated

process in both directions across the nuclear membrane (Guiochon-Mantel *et al.*, 1994), or whether import is facilitated, and export proceeds via diffusion out of the NPC (Guiochon-Mantel *et al.*, 1991; Laskey & Dingwall, 1993; Schmidt-Zachmann *et al.*, 1993). In the latter case, the nuclear export of the protein could be regulated by binding within the nucleus, a process called nuclear retention (Cohen & Paine, 1992; Paine, 1993; Laskey & Dingwall, 1993; Schmidt-Zachmann *et al.*, 1993; Vancurova *et al.*, 1993; 1994). In this process, the interaction of the shuttling protein with molecules in the nucleus would retain it in this cellular compartment, only allowing its diffusion out of the nucleus on release from this bound state. In this way, a model can be described whereby the shuttling protein binds to a specific karyophile in the cytoplasm, is transported to the nucleus in an ATP-and NLS-dependent manner and, once in the nucleus, is retained until the transported karyophile, which may become bound to non-diffusible elements, becomes disengaged.

Contrasting with the notion of passive export of proteins from the nucleus is the evidence that the nuclear export of the progesterone receptor, a shuttling protein, requires the presence of the NLS which also targets import (Guiochon-Mantel *et al.*, 1994). Furthermore, sequences from two proteins have been recently isolated that act as nuclear export signals when fused to heterologous proteins (reviewed in Gerace, 1995). The sequence LALKLAGLDI provides the nuclear export signal for protein kinase inhibitor (Wen *et al.*, 1995), while a similar sequence (LPPLERLTL) signals nuclear export for the HIV-1 Rev protein (Fischer *et al.*, 1995). Factors have been isolated that are capable of interaction with the Rev nuclear export signal in human cells (Bogerd *et al.*, 1995) and yeast (Stutz *et al.*, 1995). These factors may have a role in a more general pathway of nuclear export, as there is evidence that HIV-1 Rev-induced mRNA export proceeds via interaction with factors in common with the nuclear export of 5S rRNA and U1 snRNA (Fischer *et al.*, 1995).

1.7 5S rRNA Nuclear Transport and the Scope of This Thesis

The aim of the research described in this thesis was to investigate the mechanism whereby 5S rRNA is imported into the nucleus after storage in the cytoplasm in *Xenopus* oocytes. This thesis is divided into three sections of

experimental work focusing on different areas of the nuclear import process: the RNP status of 5S rRNA during transport; the role of the nuclear pore complex; and the involvement of cytoplasmic factors, utilising a comparative investigation with other karyophilic molecules.

Chapter Two addresses the mechanism whereby 5S rRNA passes through the nuclear membrane, and investigates whether this occurs via a passive or facilitated process through the nuclear pore complex. General inhibitors of facilitated NPC transport were used to assess their effect on the nuclear import of radiolabelled 5S rRNA microinjected into the cytoplasm of *Xenopus* oocytes. Chapter Three addresses the role of ribosomal protein L5 in the nuclear import of 5S rRNA. The nuclear import characteristics of *in vitro*-generated radiolabelled L5/5S rRNA complexes (5S RNPs) are compared with the characteristics of labelled 5S rRNA described in Chapter Two. Evidence is presented for 5S rRNA being bound in the 5S RNP during the nuclear import process, and the implications for the function of ribosomal protein L5 in signalling release from cytoplasmic storage and subsequent nuclear import are discussed. Finally, Chapter Four provides a comparison of the pathway of nuclear transport of 5S RNPs with a nuclear protein and two small nuclear RNAs (one nucleoplasmic and the other nucleolar), to determine whether common saturable components exist between the different karyophiles. The results of these studies give an indication of the nature of the 5S RNP NLS and give further evidence of a role for ribosomal protein L5 in the targeting of 5S rRNA for nuclear import.

Chapter Two

5S rRNA Nuclear Import: A Passive or Facilitated Process Through the Nuclear Pore Complex?

2.1 Introduction

As described in Chapter One, oocyte-type 5S rRNA moves between the cytoplasmic and nuclear compartments of *Xenopus* oocytes during development. In the study outlined in this chapter, the mechanism of 5S rRNA nuclear import after storage in the cytoplasm has been investigated. Part of the work described in this chapter has been published (Allison *et al.*, 1993; refer to Appendix)

2.1.1 Facilitated versus passive transport

Transport of ions and molecules across the nuclear membrane takes place exclusively through the nuclear pore complex (NPC). Movement through the NPC channel has been categorised in two ways: passive diffusion, if the molecule is below the 40 kD exclusion limit for transport through the resting NPC (Peters, 1984); and active transport that requires ATP and involves an expansion of the pore channel to allow access of larger molecules (Akey, 1990; 1992; Forbes, 1992; Hurt, 1993; Panté & Aebi, 1993; 1994; Agutter & Prochnow, 1994; Hinshaw, 1994; Davis, 1995). Recently, a re-evaluation of these terms has taken place and the definition of all ATP-requiring nuclear transport as being 'active transport' has been proposed to be too simplistic (Cohen & Paine, 1992; Paine, 1992; 1993; Vancurova *et al.*, 1993; Davis, 1995). Therefore, a third category of transport through the NPC, facilitated diffusion, must be considered.

Simple passive diffusion through the NPC is a process that is restricted by the sieve-like nature of the nuclear envelope to molecules of less than 90-100 Å in diameter. The process involves movement down the chemical gradient formed across the nuclear membrane and through the aqueous phase of the cell. Therefore, passive diffusion does not include binding to 'static' components such as the cytoskeleton and membranes, nor does it involve interaction with receptors that target the molecule for

nuclear transport. Simple passive diffusion is a relatively slow process, with less potential for regulation than processes involving receptor-ligand interactions, and does not require ATP. Nuclear transport through the process of passive diffusion can result in net nuclear (or cytoplasmic, in the case of nuclear export) accumulation, if there are retention sites for the molecule in that cellular compartment. In this way, karyophiles could 'drift' into the nucleus and remain there by binding to macromolecules or non-diffusible factors, thus maintaining the direction of the chemical gradient in the soluble phase. It appears that few molecules of physiological importance are likely to undertake nucleo-cytoplasmic transport by simple passive diffusion alone. Even some that fall within the size limit for passive diffusion through the NPC channel (assuming that there is no binding of other proteins), such as histone H1 (Breeuwer & Goldfarb, 1990) and SV40 Vp3 (Dean & Kasamatsu, 1994), are imported via a facilitated process. However, the free catalytic subunit of cAMP-dependent protein kinase microinjected into cultured cells does undergo nuclear transport in both directions by simple diffusion (Harootunian *et al.*, 1993).

Active transport has an essential requirement, either directly or indirectly, for metabolic energy in the form of ATP, while facilitated diffusion may or may not require ATP. The feature differentiating active transport from facilitated diffusion is that active transport must occur against the chemical activity gradient, while facilitated diffusion proceeds down the gradient (Cohen and Paine, 1992). Chemical activity gradients are formed by molecules in the solution within the cell; that is, the gradient does not include, for example, those molecules bound to non-diffusible elements such as the cytoskeleton. In reality, it is difficult to determine the chemical activity gradient of a molecule *in vivo*, as the proportion of the cytoplasmic and nuclear pools of the molecule that are bound or soluble are difficult to determine experimentally. Recently, Vancurova *et al.* (1993) devised a method for determination of the chemical activity gradient, and involvement of nuclear retention, in the nuclear uptake of nucleoplasmin, an abundant protein in *Xenopus* nuclei. Using oil-isolated *Xenopus* oocyte nuclei paired with either a bolus of cytoplasm or a latex bead loaded with fluorescently-labelled nucleoplasmin, they established that nucleoplasmin reached equilibrium between the bead and nucleus in an ATP-dependent manner, whereas actual concentration within the nucleus required a factor present in the bolus of cytoplasm. In addition, puncturing the nuclear membrane, thereby releasing the NPC

restriction for transport, did not alter the kinetics of nucleoplasmin uptake, suggesting that transport does not normally occur against the chemical activity gradient and that the protein accumulates in the nucleus by binding to a non-diffusible element. Therefore, nucleoplasmin import can be explained by facilitated diffusion, followed by binding within the nucleus.

Concentration of karyophiles within the nucleus could be explained entirely by facilitated diffusion and intranuclear binding, without a requirement for a motor-like transporter to move the molecule (or complex) through the NPC channel. It has been suggested that the role of proteins such as Ran, a small GTPase that has been implicated in the process of nuclear transport (refer to Chapter One, section 1.5.2.2; Kadowaki *et al.*, 1993; Melchior *et al.*, 1993; Moore & Blobel, 1993; 1994; Bischoff *et al.*, 1995a,b; Moroianu & Blobel, 1995), may be to target molecules to specific intranuclear binding sites, rather than for translocation through the NPC channel (Cheng *et al.*, 1995; Davis, 1995; Tartakoff & Schneiter, 1995). It is plausible that nuclear accumulation of proteins and RNAs is largely attributable to the presence of retention sites in the nucleus (Schmidt-Zachmann *et al.*, 1993; Vancurova *et al.*, 1993; 1994; Boelens *et al.*, 1995; Paine *et al.*, 1995), especially for those proteins involved in DNA and RNA binding (LaCasse & Lefebvre, 1995). Furthermore, the nuclear export of some shuttling proteins occurs via a diffusional pathway, with the nuclear:cytoplasmic distribution of the protein being regulated by a combination of facilitated import and nuclear retention site availability, as is the case for the nucleolar protein nucleolin (Schmidt-Zachmann *et al.*, 1993).

5S rRNA is 41 kD in size (121 nucleotides) and therefore falls within the limit for unrestricted diffusion through the resting NPC. Therefore, it is possible that the mechanism of transport of this molecule could be via either of the three mechanisms outlined above. However, a 3′ truncated 5S rRNA molecule of 98 nucleotides in length is excluded from the nucleus after cytoplasmic microinjection into *Xenopus* oocytes, suggesting that the full length 5S rRNA does not move into the nucleus by simple diffusion (Allison *et al.*, 1991; 1993). To distinguish between the possible transport mechanisms, treatments that inhibit facilitated transport through the NPC were used in the present study to assess their effect on 5S rRNA nuclear transport.

2.1.2 Temperature dependence and ATP requirement

Several criteria have been used in other studies to establish whether the nucleo-cytoplasmic transport of proteins and RNAs occurs via an active or passive mechanism. Firstly, the facilitated transport of molecules across the nuclear membrane is inhibited at 0 to 4 °C, while passive diffusion can occur at approximately 90% of the normal rate (Breeuwer & Goldfarb, 1990; Kambach & Mattaj, 1992). Reduction in nuclear transport at low temperatures is due to a general reduction in the rate of enzyme-mediated processes in chilled cells. It is therefore possible to lower the temperature of incubation for the cells under treatment and observe a reduction in active nuclear transport, while the process of passive diffusion is unaffected. In this way, chilling has been shown to inhibit the nuclear import of all karyophilic proteins studied (for reviews see: Roberts, 1989; Wagner *et al.*, 1990), as well as the export of U snRNAs (Jarmolowski *et al.*, 1994), tRNA (Zasloff, 1983), signal recognition particle RNA (He *et al.*,1994), mRNA (Dargemont & Kühn, 1992), the RNA components of Ro RNPs, Y RNAs (Simons *et al.*, 1994), and ribosomal subunits (Bataillé *et al.*, 1990).

Another means of determining whether the mechanism of passage of a macromolecule through the nuclear membrane is energy-dependent is to test whether the process continues after depletion of ATP. ATP-hydrolysing enzymes have been used in a cell-free system (Newmeyer *et al.*, 1986a,b), as well as in *Xenopus* oocytes (Bataillé *et al.*, 1990), to reduce ATP levels and to distinguish between facilitated transport and passive diffusion of protein and ribonucleoprotein particles through the nuclear membrane. ATP has been found to be an absolute requirement for the nuclear transport of RNAs and RNPs such as influenza virus RNPs (Kemler *et al.*, 1994), mRNA (Dargemont & Kühn, 1992), and ribosomal subunits (Battaillé *et al.*, 1990).

Even though the requirement for ATP in the above transport processes is well established, its exact role is unclear. ATP has been found to be required for the translocation step of passage through the NPC, but not the binding at the nuclear envelope (Newmeyer & Forbes, 1988; Richardson *et al.*, 1988). Therefore, it may be important in the activation of the machinery which enlarges the pore diameter to allow entry of the molecule, or propels the karyophile through the channel. A nucleoside

triphosphatase has been extracted from isolated rat liver nuclear envelopes which appears to have a role in the nuclear export of pre-mRNA (Agutter *et al.*, 1979; Bernd *et al.*, 1982; Schröder *et al.*, 1986a,c). Translocation through the NPC channel may involve ATP hydrolysis by this enzyme. The activity of this nucleoside triphosphatase is thought to be regulated by phosphorylation and dephosphorylation via protein kinases (Schröder *et al.*, 1986b; 1988). It has been proposed that the nuclear envelope nucleoside triphosphatase is in the form of myosin molecules, which could be integral to the NPC structure, providing an ATP-dependent contractile mechanism for the propulsion of molecules through the channel (Berrios & Fisher, 1986; Berrios, 1992). In addition, ATP has been proposed to be required for motor-driven transport of proteins and RNPs along cytoskeletal or nucleoskeletal tracks to the NPC (Rosbash & Singer, 1993). However, recent studies suggest that it is GTP hydrolysis, mediated by the small GTPase Ran and its accessory proteins, and not ATP hydrolysis that is required for translocation of molecules through the nuclear pore complex (Kadowaki *et al.*, 1993; Melchior *et al.*, 1993; Bischoff *et al.*, 1995a,b; Cheng *et al.*, 1995; Moroianu & Blobel, 1995; Schlenstedt *et al.*, 1995). The role of ATP in nuclear transport may be in the mediation of the operation of this GTPase via ATP-utilising activating proteins.

Alternatively, ATP may be required at the earlier steps in the pathway for nuclear transport. The activation of nuclear localisation signals may require ATP for the phosphorylation of sites on the molecule adjacent to the signal. Phosphorylation has been implicated in the functioning of NLSs of the SV40 large T antigen (Rihs & Peters, 1989; Rihs *et al.*, 1991; Jans & Jans, 1994), nucleoplasmin (Vancurova *et al.*, 1995) and the serum response factor, a ubiquitous nuclear transcription factor (Gauthier-Rouvière, 1995). ATP may also be required in the promotion of interactions with factors which promote nuclear targeting such as a NLS receptor or nuclear pore targeting complex (Adam & Adam, 1994; Powers & Forbes, 1994; Görlich *et al.*, 1995a,b; Imamoto *et al.*, 1995a,b,c).

Another possible role of ATP in nuclear transport, which is not necessarily mutually exclusive with the mechanisms proposed above, is its involvement in changing the conformational state of the transported molecule. This could be important in the maintenance of the nuclear targeting signal in an exposed state to

enable interaction with the nuclear transport machinery, or in the transformation of the molecule into an elongated form, thereby allowing passage through the NPC channel. Electron microscopic analysis of different *Chironomus* ribonucleoprotein particles has determined that there is a change from globular to more linear form as the particles approach the NPC (Mehlin *et al.*, 1992; 1995). There is also evidence of a role for a nuclear envelope-associated RNA helicase, which unwinds sections of double stranded RNA, in the nuclear export of mRNA (Schröder *et al.*, 1990). In addition, heat shock proteins, which act as molecular chaperones for transport into mitochondria by altering the conformation of the protein in an ATP-dependent manner, are also involved in nuclear transport (Imamoto *et al.*, 1992; Shi & Thomas, 1992; Okuno *et al.*, 1993; Yang & DeFranco, 1994).

2.1.3 Wheat germ agglutinin

Wheat germ agglutinin (WGA) is a plant lectin, or carbohydrate-binding protein that specifically binds *N*-acetylglucosamine (GlcNAc) residues (Hanover *et al.*, 1987; Holt *et al.*, 1987; Yoneda *et al.*, 1987). As described in Chapter One, the NPC contains a number of glycoproteins which are essential to its structure, with respect to the nuclear membrane, and function in nuclear transport. Several of these so-called nucleoporins have been isolated by antibodies directed against NPCs, and have been characterised as having GlcNAc residues joined by unusual O-linkages to their polypeptide backbone, at approximately 10 moles of GlcNAc per mole of protein (Holt *et al.*, 1987). WGA has a high affinity for clusters of GlcNAc residues and hence binds these proteins.

Fluorescently-labelled WGA binds to the nuclear envelope at the nuclear pore complex, giving a characteristic punctate staining pattern on the nuclear envelope (Finlay *et al.*, 1987; Yoneda *et al.*, 1987). Higher resolution images have been obtained using electron microscopy of isolated nuclear membranes labelled with gold-conjugated WGA (Akey and Goldfarb, 1989; Wilken *et al.*, 1993; Panté & Aebi., 1994), or WGA conjugated to the electron-dense ferritin molecule (Finlay *et al.*, 1987; Allen, 1990). From these images it has been determined that WGA binds to the central 'transporter' region of the NPC (Akey and Goldfarb, 1989) and at greater than 15 molecules per pore (Finlay *et al.*, 1987). In addition, Allen (1990) visualised

ferritin-labelled WGA binding to fibrous material at both cytoplasmic and nucleoplasmic pore margins in nuclei of cultured mammalian cells.

Treatment of cells with WGA has been found to inhibit the nuclear import of karyophilic proteins including nucleoplasmin in an *in vitro* system (Finlay *et al.*, 1987) and in cultured cells (Yoneda *et al.*, 1987); synthetic peptide containing the SV40 large T antigen nuclear localisation sequence in cultured cells (Wolff *et al.*, 1988); and a group of labelled proteins microinjected into *Xenopus* oocytes (Dabauvalle *et al.*, 1988). Treatment with WGA prevents the efflux of RNPs from rat nuclei (Baglia and Maul, 1983); as well as export of small nuclear RNA precursors (Neuman de Vegvar and Dahlberg, 1990), mRNA (Dargemont and Kühn, 1992), signal recognition particle RNA (He *et al.*, 1994), and ribosomal subunits (Bataillé *et al.*, 1990) in *Xenopus* oocytes. The nuclear import of the U1-U6 snRNAs in *Xenopus* oocytes is also sensitive to WGA (Fischer *et al.*, 1991; Michaud and Goldfarb, 1992). WGA-induced transport inhibition has been shown to be specific to facilitated transport and not simply a function of blocking or constraining the NPC channel, as the passive diffusion of small fluorescently-labelled dextran molecules through the NPC continues at a normal rate with this treatment (Finlay *et al.*, 1987; Yoneda *et al.*, 1987; Dabauvalle *et al.*, 1988; Wolff *et al.*, 1988). As mentioned in Chapter One (section 1.3.2), antibodies raised against the WGA-binding nucleoporins (Davis & Blobel, 1986; Snow *et al.*, 1987) also have an effect on nucleo-cytoplasmic transport, presumably by binding and thereby inactivating the nucleoporins (Featherstone *et al.*, 1988; Akey & Goldfarb, 1989; Finlay *et al.*, 1991; Michaud & Goldfarb, 1992; Yamada & Kasamatsu, 1993; Kemler *et al.*, 1994).

The *in vitro* construction of NPCs which are depleted of WGA-binding nucleoporins has provided further evidence of a role for these proteins in nucleo-cytoplasmic transport (Finlay & Forbes, 1990; see also Chapter One). In this study, nuclei were constructed in a nuclear assembly extract devoid of WGA-binding proteins. These biochemically altered NPCs were functional for diffusion, but not for the binding of nuclear localisation signals, or subsequent facilitated transport. Previous studies had determined that WGA inhibits the ATP-dependent translocation step of NPC transport, but not the initial binding step (Newmeyer & Forbes, 1988). Therefore, the inhibition of binding in biochemically altered NPCs may be due to an

indirect structural perturbation in the NPCs created without these proteins (Finlay & Forbes, 1990). However, nuclear localisation signal-dependent binding of nucleoplasmin at the NPC has been visualised at WGA-reactive fibrils that radiate from the pore to the cytoplasm (Richardson *et al.*, 1988; Allen, 1990), thus corroborating the concept of WGA-binding proteins being involved in this initial interaction (see also Rout & Wente, 1994). Furthermore, factors involved in the docking of karyophiles at the NPC, as well as the subsequent translocation step of import, have been found to directly interact with nucleoporins that are able to bind WGA (Kraemer *et al.*, 1995; Moroianu *et al.*, 1995a,b; Paschal & Gerace, 1995; Radu *et al.*, 1995a,b; Yokoyama *et al.*, 1995).

Facilitated nuclear transport is not necessarily inhibited by chilling, ATP depletion and WGA. For example, the nuclear import of the Ca^{2+}-binding protein calmodulin in tissue culture cells has been found to be inhibited by chilling and wheat germ agglutinin treatment but not by ATP depletion, demonstrating that calmodulin nuclear import proceeds via facilitated diffusion (Pruschy *et al.*, 1994). Thus, ATP may not be an essential component in all mediated pathways of nuclear transport, as facilitated diffusion can proceed in its absence (see Section 2.1.1). In summary, chilling, ATP depletion and WGA treatments are able to differentiate passive diffusion from facilitated NPC-mediated transport and, in some cases, may also be capable of distinguishing between facilitated diffusion and active transport.

2.1.4 Assays used in the present study

Microinjection of labelled molecules into *Xenopus* oocytes has been used extensively in studies of RNA/RNP nuclear transport processes (DeRobertis *et al.*, 1982; Bataillé *et al.*, 1990; Guddat *et al.*, 1990; Allison *et al.*, 1991; Fischer *et al.*, 1991; 1993; Michaud & Goldfarb, 1991; 1992; Dargemont & Kühn, 1992; Terns *et al.*, 1993; Jarmolowski *et al.*, 1994; He *et al.*, 1995; Pokrywka & Goldfarb, 1995). Oocytes are readily obtained by surgical removal from adult females and, at approximately 1.2 mm in diameter (for late-stage oocytes), they are large enough to be easily manipulated and microinjected. In addition, the nucleus can be manually separated from the cell, allowing a simple determination of nuclear transport by measurement of the cytoplasmic and nuclear pools of the molecule(s) under study. In

this investigation, the use of *in vitro*-generated, radiolabelled 5S rRNA enabled the quantification of 5S rRNA import under various treatments that inhibit facilitated transport through the nuclear pore complex.

2.2 Materials and Methods

Unless otherwise stated, general reagents used in these studies were obtained from BDH (Poole, England) and electrophoresis reagents were obtained from Boehringer Mannheim (N.Z. Ltd., Auckland, New Zealand). All chemicals were of Analytical or Molecular Biology Grade.

2.2.1 *In vitro* synthesis of ^{32}P-5S rRNA

The oocyte-type 5S rRNA gene template, pXlo-wt (Romaniuk *et al.*, 1987), was provided by Dr P.J. Romaniuk (University of Victoria, Victoria, British Columbia, Canada). Internally-labelled oocyte-type ^{32}P-5S rRNA was synthesised *in vitro* from pXlo-wt linearised with *Dra I* (Boehringer Mannheim). Transcription reactions were performed using 50 U T7 RNA polymerase (Epicentre Technologies: Intermed Scientific Ltd., Auckland, New Zealand) and 100 µCi [α-^{32}P]GTP (3000 Ci/mmole; Amersham Australia Pty. Ltd or DuPont NEN: Life Technologies Ltd., Auckland, New Zealand) in a 20 µl reaction containing transcription buffer (Epicentre), 10 m*M* dithiothreitol, 40 U RNasin (Promega: Pacific Diagnostics Pty. Ltd., Auckland, New Zealand), 200 µ*M* ATP, CTP and UTP, 40 µ*M* GTP and 1 µg DNA template. The mixture was incubated for 1 h at 37°C followed by treatment with 1 U RNase-free DNase I (Boehringer Mannheim) for 15 min at 37°C. After incubation, 130 µl UB Blue (175 m*M* NaCl, 5 m*M* Tris, pH 7.4, 5 m*M* EDTA, 0.5% sodium dodecylsulphate [SDS], 0.05% methylene blue, 7 *M* urea) and 1 µg glycogen (Boehringer Mannheim) were added. The samples were purified by extraction with an equal volume of 1:1 phenol:chloroform (24:1 chloroform:isoamyl alcohol), followed by extraction with an equal volume of chloroform, and precipitated with 2 vol ethanol for 30 min on ice. The RNA was collected by centrifugation at 10 000 x *g* for 20 min at room temperature, and the pellet washed with 300 µl 70% ethanol. The dried pellet was

resuspended in TE (10 m*M* Tris-HCl, pH 7.6, 1 m*M* EDTA) and stored at −80°C. The RNA was quantified using DNA Quik STRIPs (Eastman Chemical Co., New Haven, CT, USA), according to the manufacturer's instructions. The typical yield was 2 µg per reaction.

2.2.2 Surgical removal of oocytes and preparation for microinjection

Xenopus laevis (Xenopus I, Ann Arbor, MI, USA) were reared on a diet of cockroaches and salmon pellets. Adult females were anaesthetised by immersion in ice-water for 30 min followed by placement on ice for a further 30 min. A 1 cm incision was made in the skin of the lower abdomen (on one side above the leg) followed by an incision through the muscle layer to expose the ovarian tissue. A lobe of ovary was removed with sterile scissors and placed in TNE solution (0.15 *M* NaCl, 0.05 *M* Tris-HCl, pH 8.0, 1 m*M* EDTA). The incision was sutured with 4/0 coated Vicryl dissolvable sutures (Ethicon, UK), with 2-3 stitches in the muscle layer and the same number in the skin. The frog was then maintained on ice for 10 min prior to placing in ice water. When the animal had started movement, it was placed back into its tank, maintained with running water at 18°C. All procedures in the surgical removal of oocytes from the frogs had been approved by the University of Canterbury Animal Ethics Committee.

After rinsing the tissue three times with TNE to remove blood, EDTA was removed by rinsing in phosphate buffered saline (PBS; 68 m*M* NaCl, 1.3 m*M* KCl, 4.0 m*M* Na_2HPO_4, 0.7 m*M* KH_2PO_4). The oocytes were then separated by treatment in 1 mg/ml collagenase (Type I, Sigma Chemical Co., St Louis, MO, USA; made up in 0.1 *M* sodium phosphate, pH 7.4) for 30 min with constant end-over-end rotation. The separated oocytes were next washed twice with PBS and twice with O-R2 medium (82.5 m*M* NaCl, 2.5 m*M* KCl, 1.0 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 1.0 m*M* Na_2HPO_4, 5.0 m*M* HEPES [*N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulphonic acid], 3.8 m*M* NaOH), and stored in O-R2 at 18°C in culture dishes. The O-R2 was changed daily and any unhealthy oocytes (those with uneven pigmentation giving a mottled appearance) were removed.

2.2.3 Microinjection

Microinjection needles were drawn from 1 mm OD glass capillary tubes (Clark Electromedical, England) using a Narishige (Japan) micropipette puller (model PW-6), and the tip was broken to produce a needle diameter of approximately 20 µm. Microinjections were performed using a PV830 Pneumatic PicoPump (World Precision Instruments Inc., New Haven, CT, USA.) and micromanipulator (model M3301). The samples to be injected were loaded under vacuum and delivered under nitrogen gas pressure of approximately 35 psi (varied according to the solution being injected). Calibration of the injection amount was performed by measuring the diameter of the drop ejected and calculating its volume. The volume delivered was adjusted by varying the time (in msec) the pressure was activated.

Stage V to VI oocytes were selected based on the classification of Dumont (1972). Stage V oocytes were defined as 1000 to 1200 µm in diameter with a pigmented animal hemisphere. Stage VI oocytes were defined as 1200 to 1300 µm in diameter with a distinct unpigmented equatorial band. To stabilise oocytes during the microinjection process they were placed in culture dishes to which 900 µm nylon mesh had been attached. Twenty to 50 nl of sample was injected into the oocyte cytoplasm at the equatorial region of the vegetal hemisphere, with the oocyte just covered with O-R2 medium. To ensure the simultaneous initiation of nuclear transport for all treatments, dishes of oocytes were placed on ice until all microinjections were completed before placing at 18°C to begin the assay.

2.2.4 Nuclear dissections and RNA extraction

For ^{32}P-5S rRNA nuclear transport assays, nuclei were manually dissected in ice cold 1% trichloroacetic acid with watchmaker's forceps. The nucleus was extruded from the oocyte after making a single tear at the animal pole with one pair of forceps and applying pressure from the vegetal hemisphere with another pair of forceps. Nuclei were manually cleared of any adhering cytoplasmic material with the forceps, and the enucleated cytoplasm and clean isolated nucleus were transferred to separate microfuge tubes on ice. Total RNA was extracted from five pooled nuclear and cytoplasmic fractions per sample.

RNA was extracted from nuclear and cytoplasmic oocyte fractions using a potassium acetate method modified from Peppel and Baglioni (1990). To enhance the efficiency of the extraction of RNA from the nuclear fractions, the total amount of RNA was increased by the addition of two uninjected 'carrier' oocytes. The samples were homogenised in Solution 1 (2% SDS, 200 m*M* Tris-HCl, pH 7.5, 1m*M* EDTA) by pipetting in and out of a Gilson pipette tip and mixed for 5 s using a Vortex mixer at high speed. 150 µl of ice-cold Solution 2 (42.9 g potassium acetate and 11.2 ml glacial acetic acid in 100 ml) was added and the samples mixed for 10 s using a Vortex mixer, at low speed to prevent shearing the chromosomal DNA that could subsequently contaminate the RNA. The samples were then placed on ice for 2 min to precipitate the DNA and proteins, and these were removed by centrifugation at 10 000 x *g* for 5 min at room temperature. The RNA samples were further purified by extracting the clear supernatant twice with 300 µl of chloroform:isoamyl alcohol (24:1) and precipitated with 650 µl of isopropanol at -20°C overnight. The RNA was collected by centrifugation at 10 000 x *g* for 25 min at room temperature and the pellet washed with 1 ml of 70% ethanol. The RNA pellet was air dried for 5-10 min and dissolved in 10 µl formamide-dye loading buffer (95% deionised formamide, 0.05% xylene cyanol, 0.05% bromophenol blue) for polyacrylamide gel electrophoresis.

2.2.5 Gel electrophoresis and autoradiography

RNA samples were run on 8% polyacrylamide gels containing 8 M urea in TBE (89 m*M* Tris, 89 m*M* boric acid, 2.7 m*M* EDTA). The samples were heated at 100°C for 3 min to ensure denaturation, and placed immediately on ice prior to loading on the gel. Electrophoresis was carried out in TBE at 600 V (constant voltage) for a 20 cm long gel of 0.75 mm in thickness (Protean IIxi; BioRad Pty Ltd, Auckland, New Zealand) for approximately 1 h, until the xylene cyanol and bromophenol blue dye bands were 5 cm and 10-11 cm from the origin, respectively. After electrophoresis, gels were dried at 80°C for 2 h under vacuum, and exposed to X-ray film (Hyperfilm, Amersham) between intensifying screens (Kodak X-omatic Regular) at -80°C for 1-7 days. The intensity of bands on autoradiographs was determined by densitometry using a Zeineh analytical hand-held scanning densitometer and Biomed Image Analysis Software (Advanced American Biotechnology, Fullerton, CA, USA).

2.2.6 ATP depletion, WGA and chilling assays

For ATP depletion assays, oocytes were preinjected with 50 nl of 1 U/µl apyrase (grade VIII; Sigma Chemical Co.) in PBS to give an intracellular concentration of 100 U/ml. After 30 min incubation at 18°C, oocytes were injected with ^{32}P-labelled 5S rRNA at 0.02 pmole per oocyte. After an additional incubation for 6 h at 18°C, the assay for nuclear import was performed as described above. To verify the loss of cellular ATP, oocyte ATP levels were measured using a CLS ATP Bioluminescence Kit (Boehringer Mannheim). Single oocytes were homogenised in boiling 20 m*M* HEPES, pH 7.5, and incubated for 5 min at 100°C. Samples were then diluted 1:500 in ddH_2O. A 500 µl sample was added to an equal volume of luciferase extract immediately prior to measurement of luminescence using an SAI ATP photometer (model 2000). For each sample, the mean was taken from four consecutive integrating counts of 60 s.

Wheat germ agglutinin (WGA; Sigma Chemical Co.) was dissolved in PBS at concentrations ranging from 0.25 to 1 mg/ml. WGA (50 nl) or PBS (as a control) was cytoplasmically injected into oocytes. After incubation at 18°C for 3 h, oocytes were injected with ^{32}P-labelled 5S rRNA, incubated for a further 18 to 26 h, and analysed for nuclear import as described above. As a control for the specificity of the WGA treatment, 50 nl of 1 mg/ml WGA mixed with 500 m*M* *N*-acetylglucosamine (Sigma Chemical Co.) was cytoplasmically injected into oocytes prior to the injection of ^{32}P-5S rRNA.

For the chilling assays, oocytes were injected with ^{32}P-5S rRNA and incubated for 6 h on ice (with the ice container placed at 4 °C) or at 18 °C, or for 6 h on ice followed by 16 h at 18 °C and 18 h at 18 °C as a control. After incubation, nuclei were manually dissected and the assay for nuclear import performed as described above.

2.2.7 FITC-dextran controls

As a control to demonstrate that chilling and WGA treatments were not affecting the process of passive diffusion through the nuclear pore, fluorescein isothiocyanate

(FITC)-labelled dextrans were injected into the oocyte cytoplasm immediately prior to incubation on ice or 3 h after microinjection of WGA. Oocytes were injected with 50 nl of 100 mg/ml FITC-dextran of 10 kD and 150 kD in size (Sigma Chemical Co.). After an 18 h incubation, nuclei were manually dissected in nucleus isolation buffer (25 m*M* Tris-HCl, pH 8.0, 10% glycerol, 5 m*M* $MgCl_2$, 2 m*M* dithiothreitol). The isolated nuclei were placed in the depression of a microscope slide in nucleus isolation buffer, and viewed under the fluorescence microscope to enable detection of FITC-dextran that had diffused into the nucleus.

2.3 Results

2.3.1 ^{32}P-5S rRNA microinjected into the oocyte cytoplasm accumulates in the nucleus over time

To establish the kinetics of import of microinjected oocyte-type 5S rRNA in my hands, I performed the assay for nuclear import over time. As had been previously demonstrated for HeLa cell 5S rRNA (DeRobertis *et al.*, 1982) and for oocyte-type 5S rRNA (Allison *et al.*, 1991), ^{32}P-5S rRNA injected into the cytoplasm of *Xenopus* oocytes was imported into the nucleus (Fig. 2.1). Nuclear accumulation reached steady-state levels after 8 to 10 hours at approximately 10% nuclear. The results shown in Fig. 2.1 (and figures showing subsequent nuclear transport assays) are from a batch of oocytes from a single frog.

2.3.2 Wheat germ agglutinin

To establish whether the nuclear import of ^{32}P-5S rRNA occurs via a facilitated or passive mechanism through the nuclear pore complex, treatments that inhibit facilitated transport were used. The first criterion for facilitated transport that was tested was sensitivity to treatment with WGA. Cytoplasmic microinjection of WGA (at an intracellular concentration of 0.1 mg/ml) three hours prior to ^{32}P-5S rRNA injection significantly inhibited ^{32}P-5S rRNA nuclear import (Fig. 2.2 A, cf. lanes 2 and 4). This effect was reversed by co-injection of N-acetylglucosamine (lane 6),

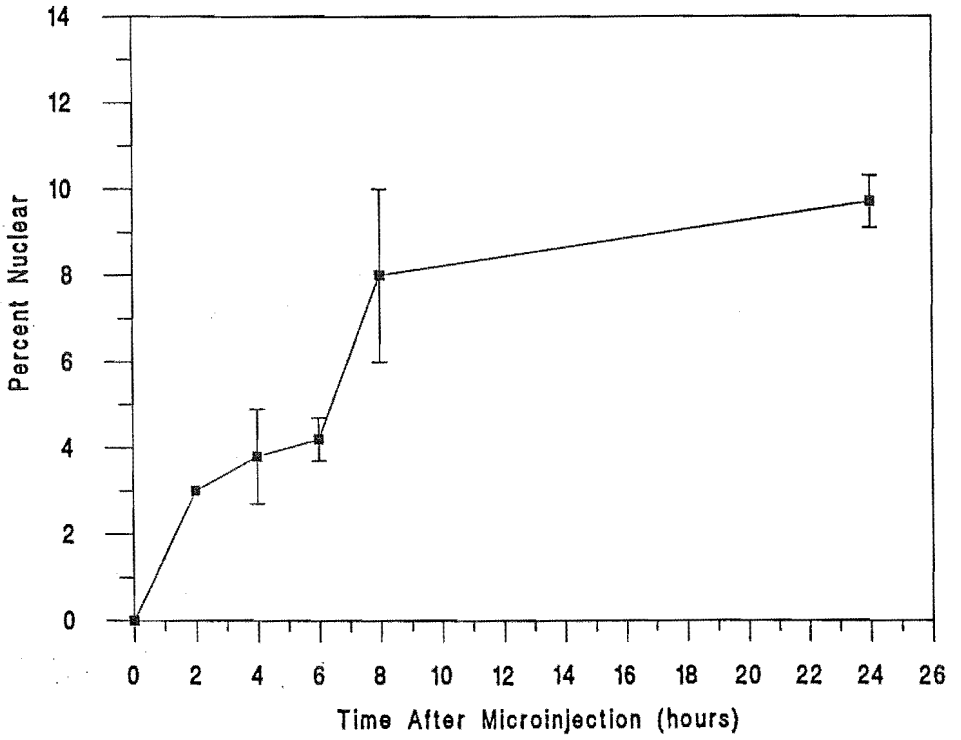
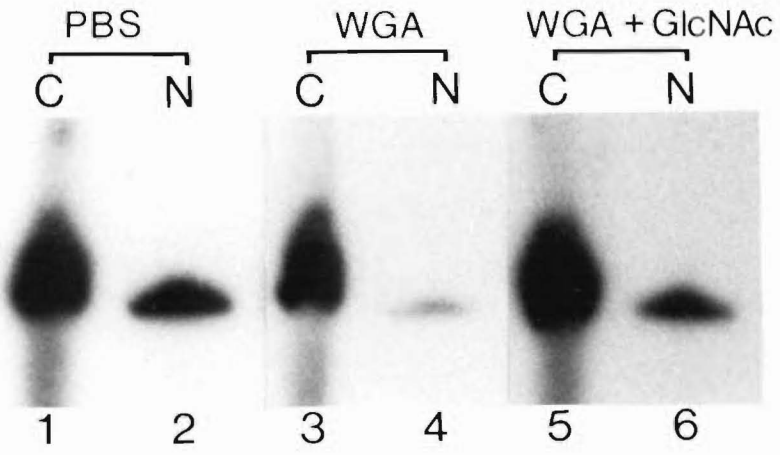


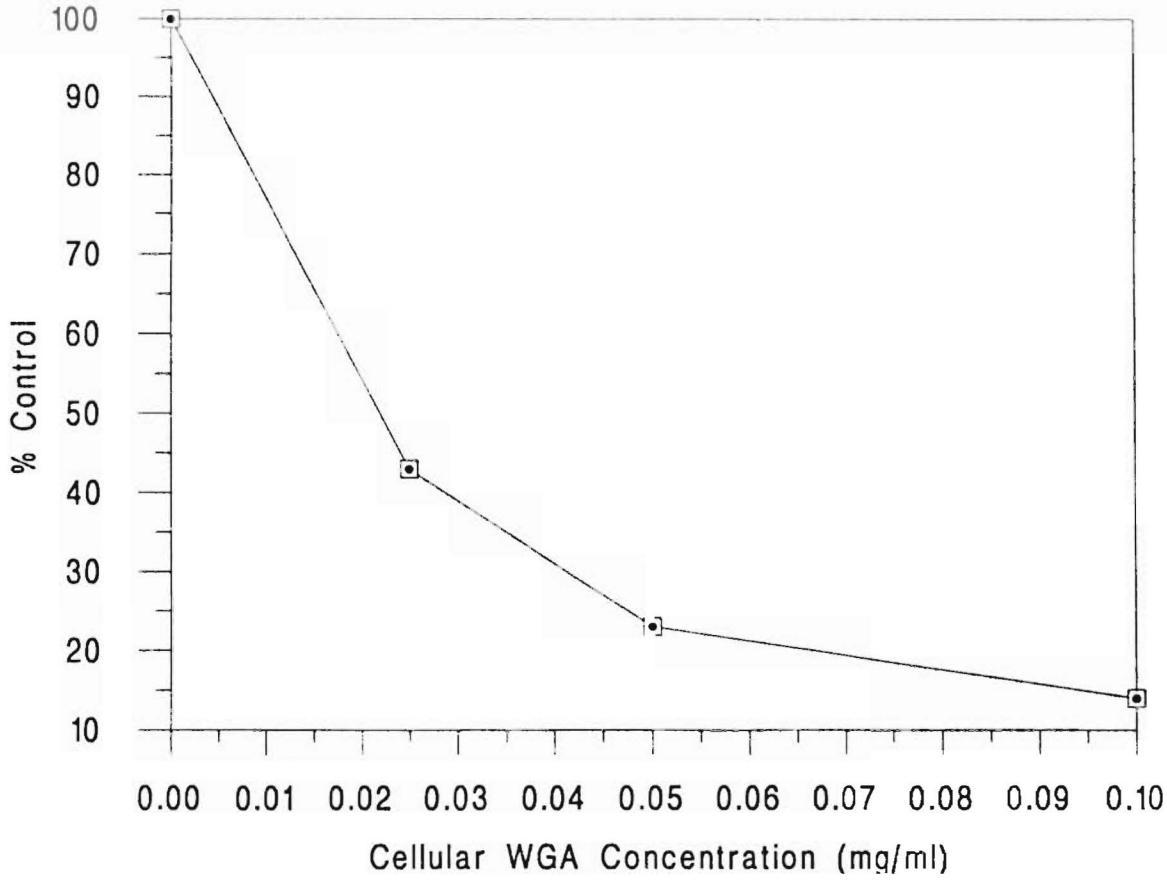
Figure 2.1: The nuclear accumulation of microinjected oocyte-type ^{32}P-5S rRNA.
In vitro-synthesised ^{32}P-5S rRNA was microinjected into the oocyte cytoplasm to a concentration of approximately 0.02 pmoles per oocyte. After incubation at 18°C for the time intervals indicated, the oocytes were manually dissected and RNA extracted from five pooled nuclear and cytoplasmic fractions. The samples were subjected to electrophoresis on denaturing 8% polyacrylamide gels containing 8 M urea, followed by autoradiography. The percentage of radioactivity in the nucleus was quantified by densitometry of the autoradiographs. Each point represents the mean from three replicate samples of five oocytes. The bars indicate the standard error of the means.

Figure 2.2: The effect of WGA on the nuclear import of 5S rRNA.
(A) Oocytes were preinjected with WGA at a cellular concentration of 0.1 mg/ml (lanes 3 and 4), or with WGA (0.1 mg/ml) and 50 mM *N*-acetylglucosamine (WGA + GlcNAc; lanes 5 and 6). PBS was injected as a control (lanes 1 and 2). After incubation for 3 h at 18°C, ^{32}P-labelled 5S rRNA was injected into the cytoplasm and the oocytes were incubated for a further 18 h. The analysis for nuclear import was then performed as described in the legend to Fig. 2.1.
C, cytoplasmic fractions; N, nuclear fractions.
(B) Dose-dependent inhibition of 5S rRNA nuclear import. Oocytes were preinjected with 50 nl of WGA at various concentrations. The final intracellular concentration of WGA is denoted on the *x* axis. After 3 h incubation, ^{32}P-5S rRNA was injected and nuclear import assayed after 18 h as described in the legend to Fig. 2.1. The percentage of radioactivity in the nucleus was quantified by densitometry of the autoradiographs. Import in the presence of WGA is expressed as a percentage of the import of ^{32}P-5S rRNA in control oocytes preinjected with PBS only (% Control).
(C) [over page] The effect of WGA on the passive diffusion of a small dextran into the oocyte nucleus. FITC-labelled dextran of 10 000 MW was cytoplasmically injected into oocytes that had been preinjected with PBS as a control, or WGA (at 0.1 mg/ml). Nuclei were manually dissected after 18 h incubation and the presence of the fluorescent label was detected by fluorescence microscopy. Nuclei from both treatments are shown with a combination of bright-field and fluorescence lighting to show the outline of the nucleus, as well as with florescence only.

A

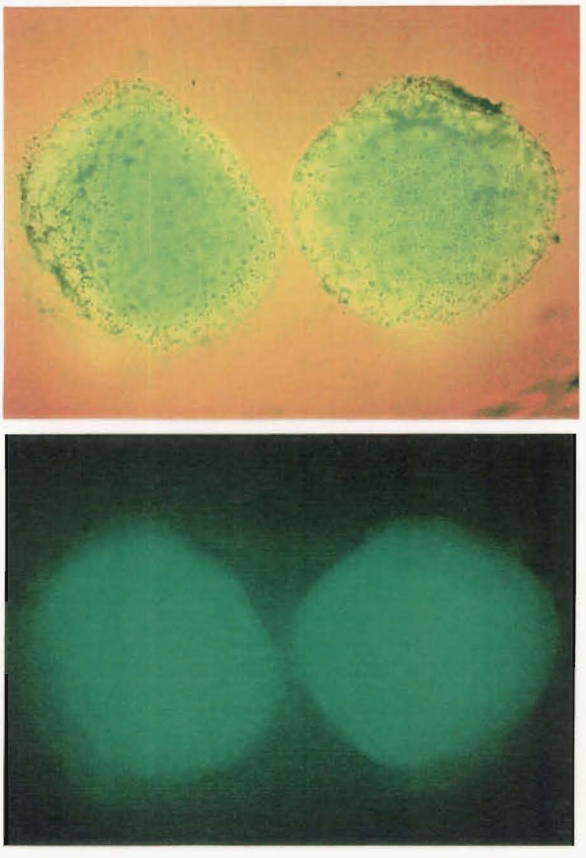

PBS
WGA
WGA + GlcNAc
C
N
C
N
C
N
1
2
3
4
5
6

B

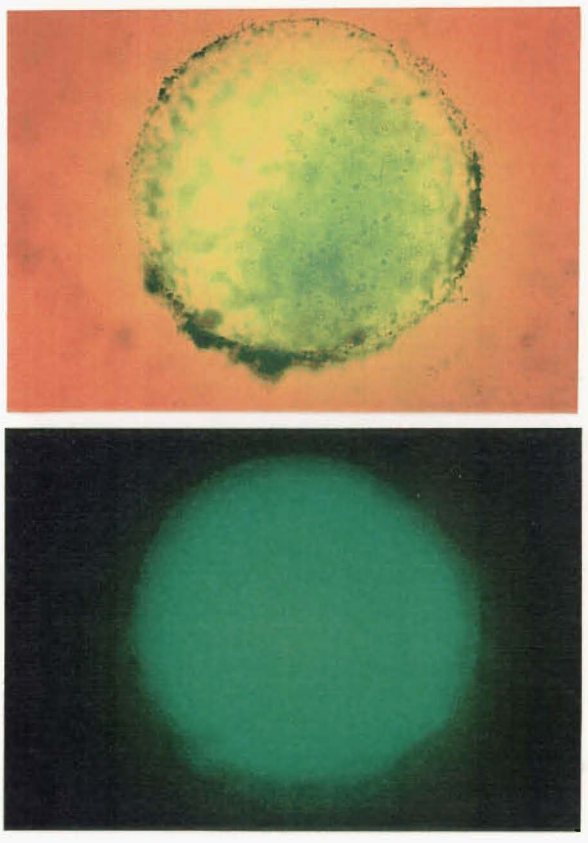

100
90
80
70
60
50
40
30
20
10
% Control
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.10
Cellular WGA Concentration (mg/ml)

C

PBS



WGA



which competes with the NPC proteins for binding to WGA. The extent of nuclear transport inhibition was dependent on the concentration of WGA injected, with an 86% reduction in nuclear accumulation at a cellular concentration of 1 mg/ml WGA (Fig. 2.2 B).

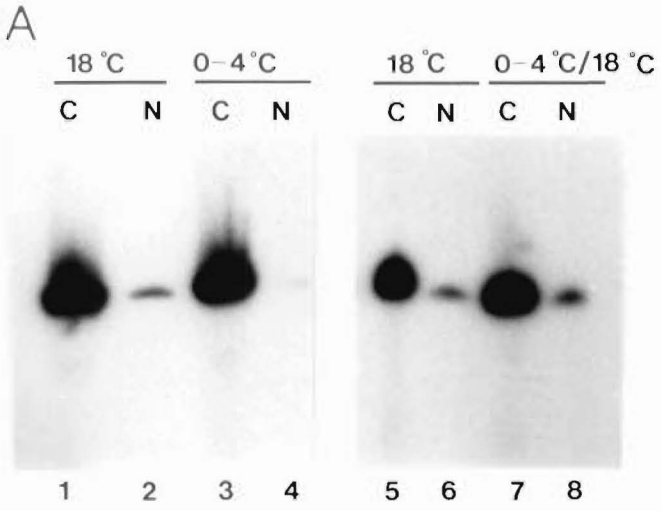
To establish that the treatment with WGA only affected facilitated transport, and was not acting by the occlusion of the NPC, WGA-treated oocytes were cytoplasmically injected with a fluorescein isothiocyanate (FITC)-labelled dextran of 10 kD in size (within the 30-40 kD size limit for passive diffusion through the nuclear pore complex). FITC-dextran was present in isolated nuclei from oocytes injected with either WGA or PBS only (Fig. 2.2 C), indicating that the process of passive diffusion was not affected. FITC-dextran of 150 kD, larger than the size limit for passive diffusion through the NPC, remained excluded from the nucleus with both treatments (data not shown). These results demonstrate that the nuclear membrane retains its characteristics as a sieve-like barrier during the assay and that the 10 kD FITC-dextran was not simply binding at the outside of the nuclear membrane without moving into the nucleus proper. This control has not previously been reported for *Xenopus* oocytes, and thus these results augment those obtained in cultured somatic cells (Dabauvalle *et al.*, 1988; Finlay & Forbes, 1990).

2.3.3 Chilling

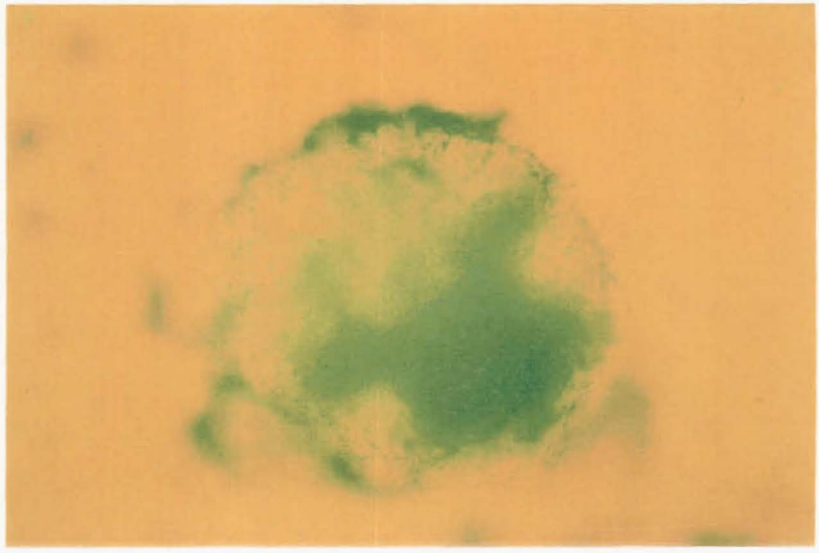
An additional criterion for mediated transport is its inhibition in chilled cells. The nuclear import of cytoplasmically microinjected ^{32}P-5S rRNA was inhibited by the incubation of oocytes on ice, at 0-4°C (Fig 2.3 A, cf. lanes 2 and 4). ^{32}P-5S rRNA nuclear transport competence was restored when the oocytes were subsequently incubated at 18°C (cf. lanes 6 and 8), suggesting that chilling blocked RNA nuclear import by inhibiting the activity of specific components of the transport machinery, rather than by preventing import by way of non-specific cellular damage. These results indicated that 5S rRNA import proceeds via a facilitated process, which is inhibited when enzyme and receptor binding activity is reduced.

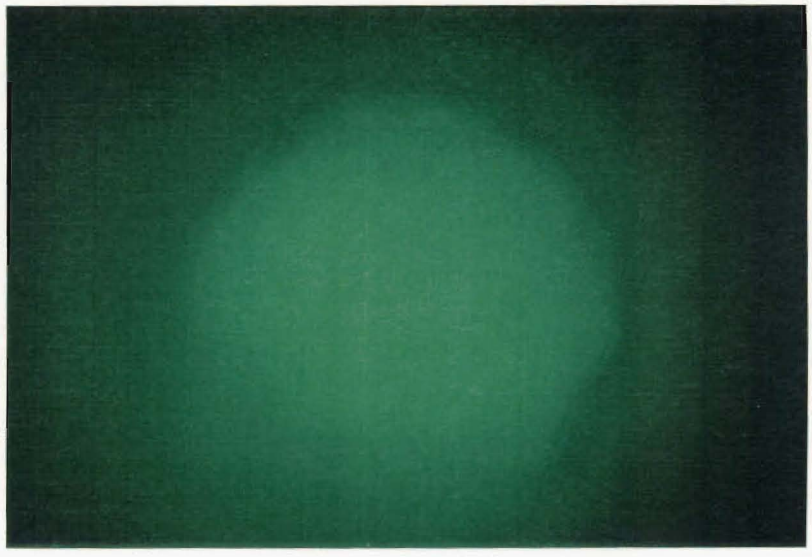
To establish that the chilling treatment was only affecting a facilitated process, the FITC-labelled dextrans were cytoplasmically injected into oocytes. Chilling

Figure 2.3: The effect of chilling on the nuclear import of 5S rRNA.
(A) ^{32}P-5S rRNA was cytoplasmically injected into oocytes. The oocytes were incubated at either 18°C for 6 h (lanes 1 and 2), 0 to 4°C for 6 h (lanes 3 and 4), 18°C for 18 h (lanes 5 and 6), or 0 to 4°C for 6 h followed by 18°C for 12 h (lanes 7 and 8). After incubation, oocytes were manually fractionated into cytoplasmic (C) and nuclear (N) compartments, and the analysis for nuclear import performed as described in the legend to Fig. 2.1.
(B) [over page] The effect of chilling on the passive diffusion of a small dextran into the nucleus. Oocytes were cytoplasmically injected with FITC-labelled dextran of 10 000 MW. After incubation at 0 to 4°C for 8 h, nuclei were manually dissected and viewed under the fluorescence microscope. The nucleus in this example is shown both with a combination of bright-field and fluorescence lighting, as well as fluorescence only.


A
18 °C
0–4 °C
18 °C
0–4 °C/18 °C
C
N
C
N
C
N
C
N
1
2
3
4
5
6
7
8

B





treatment did not affect the passive diffusion of the 10 kD into the nucleus (Fig 2.3 B), and the 150 kD dextran remained excluded from the nucleus (data not shown).

2.3.4 ATP depletion

The final criterion used to establish the passive or facilitated nature of 5S rRNA nuclear import was sensitivity to ATP depletion. Active transport through the NPC has an absolute requirement for ATP, and facilitated diffusion may have an ATP requirement. Microinjection of the ATP-hydrolysing enzyme apyrase (to an intracellular concentration of 100 U/ml) reduced the cellular concentration of ATP from 1-2 mM to 20 µM within 30 minutes, and ATP levels remained low for 24 hours after injection (Fig 2.4 A). As shown in Fig. 2.4 B, depletion of oocyte ATP levels by prior injection of apyrase had a significant inhibitory effect on the nuclear transport of cytoplasmically injected ^{32}P-5S rRNA (cf. lanes 2 and 4). The autoradiograph was deliberately over-exposed to demonstrate the absence of detectable levels of ^{32}P-5S rRNA in the nuclear fraction of ATP-depleted oocytes. The results of this experiment demonstrate that the nuclear import of 5S rRNA occurs via an ATP-requiring process.

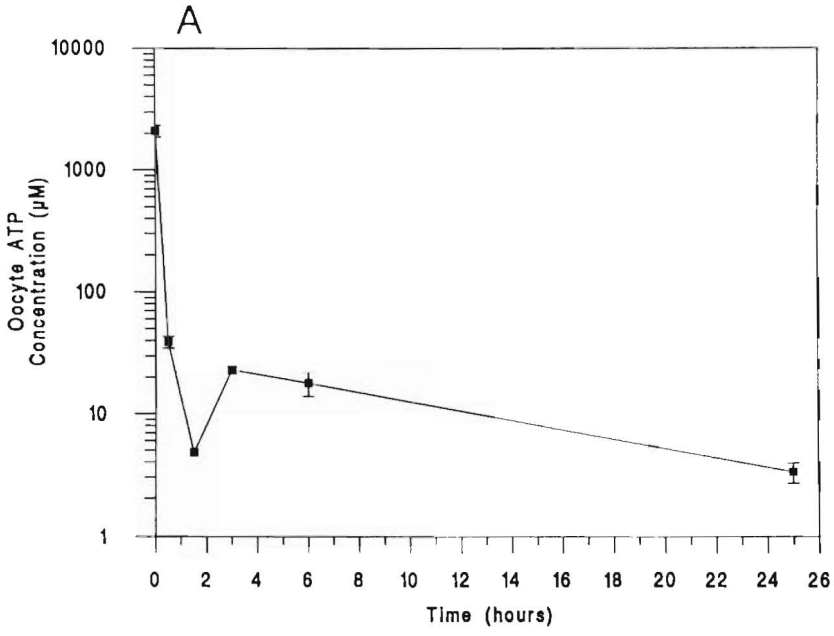
2.4 Discussion

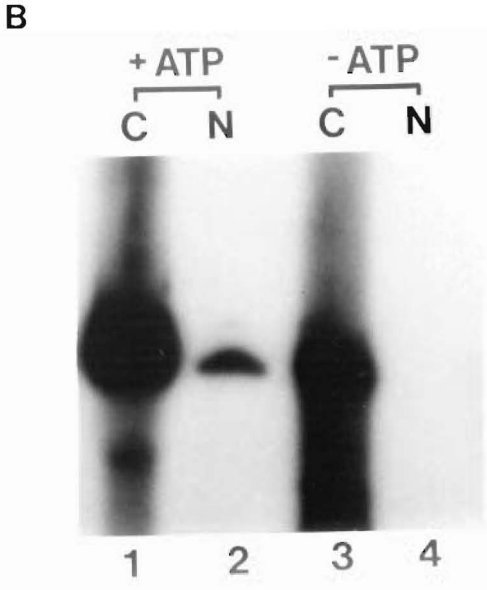
The results presented here demonstrate that the transport of oocyte-type 5S rRNA from cytoplasm to nucleus in *Xenopus* oocytes occurs via a facilitated mechanism through the nuclear pore complex. This process is temperature-dependent, requires ATP, and is inhibited by binding of WGA to nuclear pore complex proteins. In its sensitivity to these treatments, the nuclear import of 5S rRNA is similar to other small RNAs that undergo transport into the nucleus (Fischer *et al.*, 1991; Michaud & Goldfarb, 1992).

2.4.1 The nuclear accumulation of 5S rRNA

5S rRNA microinjected into the cytoplasm accumulates in the nucleus over time. Between 8 and 10 hours after microinjection, the rate of accumulation begins to diminish, resulting in a steady level of approximately 10% nuclear. This level of net

Figure 2.4: The effect of ATP depletion on the nuclear import of 5S rRNA.
(A) The effect of microinjected ATP-depleting enzymes on oocyte ATP levels. Oocytes were cytoplasmically injected with apyrase (to a cellular concentration of 100 U/ml). After incubation at 18°C for the times indicated, oocytes were individually homogenised and processed for ATP quantification using the luciferase enzyme system (see Section 2.2). Bars indicate the standard error of the means from four sample replicates.
(B) Oocytes were injected with apyrase to a cellular concentration of 100 U/ml (-ATP; lanes 3 and 4), or PBS as a control (+ ATP; lanes 1 and 2). After incubation for 30 minutes at 18°C, ^{32}P-5S rRNA was injected into the cytoplasm, and the oocytes were incubated for a further 6 h at 18°C. The analysis for nuclear import was then performed as described in Fig. 2.1. C, cytoplasmic fractions; N, nuclear fractions.


A
10000
1000
100
10
1
Oocyte ATP Concentration (μM)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)


B
+ ATP
- ATP
C
N
C
N
1
2
3
4

nuclear accumulation is half that previously reported for oocyte-type 5S rRNA injected into the cytoplasm of *Xenopus* oocytes (Allison *et al.*, 1991), probably reflecting the variability in the rate of nuclear import between batches of oocytes due to differences in age and developmental stage. This effect is also demonstrated in the results of nuclear import assays presented in this thesis, where net nuclear import of 5S rRNA after 24 hours varied between 8 and 16% (cf. Figs 2.1, 4.1, 4.2, and 4.3 B).

The extent of 5S rRNA import is low compared with other karyophiles. For example, HeLa U2 snRNA and *Xenopus* U1 snRNA were found to be approximately 60% nuclear 8 hours after injection into the oocyte cytoplasm (Michaud & Goldfarb, 1991; 1992), and the protein nucleoplasmin was found to be rapidly imported into oocyte nuclei reaching approximately 16% nuclear only one hour after its cytoplasmic injection (Michaud & Goldfarb, 1992). The low amount of 5S rRNA nuclear accumulation with respect to other karyophiles is likely to represent the complex developmental regulation of its sub-cellular localisation (described in Chapter One). Nuclear proteins are imported into the nucleus after their translation, to take part in activities such as transcription, and the U snRNAs are imported to their nuclear site of function after their assembly into U snRNPs in the cytoplasm. In contrast, the movement of oocyte-type 5S rRNA into the nucleus represents the formation of a steady-state between the import of 5S rRNA for ribosome assembly and its storage as part of storage RNPs or 80S ribosomes in the cytoplasm. Therefore, nuclear accumulation of 5S rRNA is regulated by the amount bound in import-competent RNPs (probably 5S RNPs) at any given time. The levelling of the rate of 5S rRNA nuclear accumulation may also be due in part to the saturation of some component (or components) at some stage of the 5S rRNA nuclear transport pathway.

2.4.2 The effect of chilling, ATP depletion and WGA on 5S rRNA nuclear import

In its response to the treatments used in this study, 5S rRNA nuclear import is similar to the import and export of other small RNAs (see Section 2.1), as well as the nuclear export of 5S rRNA (Jarmolowski *et al.*, 1994) and 5S rRNA-containing RNPs (Featherstone *et al.*, 1988; Battaillé *et al.*, 1990). It appears that most nuclear molecules, including proteins that fall within the passive diffusion limit for NPC

transport (Breeuwer & Goldfarb, 1990; Dean & Kasamatsu, 1994), as well as some ions (Mazzanti *et al.*, 1994), move across the nuclear membrane by a facilitated, nuclear pore-associated process that can be inhibited by chilling, ATP-depletion and WGA.

The amount of WGA required to inhibit 5S rRNA nuclear import is comparable to the concentration of WGA shown to inhibit the nuclear import of U6 snRNA, but 20 times less than the concentration required to inhibit U1 snRNA and U5 snRNA to the same degree (Fischer *et al.*, 1991; Michaud & Goldfarb, 1992). This lower WGA concentration is also sufficient to significantly inhibit the nuclear import of karyophilic proteins (Daubauvalle *et al.*, 1988; Kambach & Mattaj, 1992). As the actual mechanism of action of WGA is yet to be determined, the reason for this differential inhibition is unknown. It is possible that the action of WGA is not specific for nuclear pore activity, as there may be further sites for WGA binding within the cytoplasm which could indirectly affect nuclear transport; however, recent studies do not support this model. For example, Sterne-Marr *et al.* (1992) determined that the depletion of cytosolic WGA-binding proteins had no effect on nuclear protein import, demonstrating that WGA inhibition of transport was due to interaction with a component of the nuclear transport machinery.

The inhibitory effect of WGA on nuclear transport is likely to be due its interference with the binding of nuclear transport factors to the NPC. WGA-reactive nucleoporins have been found to interact with both the component of the nuclear pore targeting complex involved with docking at the nuclear pore (Moroianu *et al.*, 1995a,b; Paschal & Gerace, 1995; Radu *et al.*, 1995a,b), and with Ran, which is involved in later steps of the nuclear transport process (Yokoyama *et al.*, 1995). Therefore, WGA-inhibition of 5S rRNA nuclear import could be due to the failure of a 5S rRNA-containing nuclear pore targeting complex to interact with the NPC during its docking or at a subsequent step involved with translocation.

Chilling and ATP-depletion treatments have an effect on a variety of processes within the oocyte that could potentially have an impact on 5S rRNA nuclear transport. One possibility would be that the formation of transport-competent 5S rRNA-containing RNPs is inhibited by chilling. However, this is unlikely since 5S rRNA-

protein interactions such as the assembly of microinjected 5S rRNA into 7S RNPs can still occur at 0 to 4°C (Allison *et al.*, 1993). The inhibition of 5S rRNA nuclear import at reduced temperatures may be the result of either a reduced interaction with factors such as a nuclear localisation signal receptor or a nuclear pore targeting complex (Imamoto *et al.*, 1995 a,b,c), or in the reduction of activity of a motor involved in translocation through the cytoplasm or at the nuclear pore. Likewise, the ATP requirement for 5S rRNA nuclear import may be prior to or during transit through the nuclear pore complex. ATP could even be utilised subsequent to passage through the nuclear membrane, in the targeting of 5S rRNA to the nucleolus, or other intranuclear retention sites. ATP has been found to be a requirement for the intranuclear movement of a nucleolar protein No38 (Wu *et al.*, 1995b); however, other studies suggest that it is GTP hydrolysis that is required for nucleolar localisation (Finch *et al.*, 1993; Cheng *et al.*, 1995).

2.4.3 Facilitated transport of 5S rRNA

Inhibition of 5S rRNA nuclear import by chilling, ATP depletion and WGA eliminated the mechanism of passive diffusion as a possible mechanism for 5S rRNA import, but were unable to definitively distinguish between the possibilities of facilitated diffusion and active transport. Therefore, for the purposes of this study, I will refer to active transport and facilitated diffusion under the general term 'facilitated transport', meaning a transport process involving some kind of receptor-ligand interactions and a requirement for either physiological temperatures and/or ATP. For the purposes of this study the important feature of the transport process is not whether it occurs via facilitated diffusion or active transport, but that it is facilitated and receptor-mediated rather than passive. Intuitively, it would appear to be advantageous to utilise a facilitated process, which is more readily regulated and faster than passive diffusion alone (Paine, 1993). This would be especially important in the import of a ribosomal component, which may need to be coordinated with the import of ribosomal proteins. The import of oocyte-type 5S rRNA in *Xenopus* oocytes has been found to occur at the stage of development when ribosomal proteins are also being imported, and the other ribosomal RNAs are being synthesised (Allison *et al.*, 1991, and references therein). Furthermore, it is likely that 5S rRNA is imported into the nucleus specifically bound with protein as an RNP, which would

result in a complex too large to pass through the resting pore channel, thereby necessitating a facilitated process.

An important feature of 5S rRNA nuclear import that is not directly addressed in the experiments described in this Chapter, is the implications of RNA-protein interactions on the sub-cellular localisation of 5S rRNA. These interactions may serve to maintain 5S rRNA in the correct cellular compartment for the particular developmental stage of the oocyte by masking or exposing putative nuclear transport signals, and/or signalling retention in the cytoplasm or nucleus. Signals provided by bound proteins are likely to account for both retention in the cytoplasm for storage and subsequent nuclear accumulation at a later developmental stage. 5S rRNA binds with ribosomal protein L5 in a 5S RNP complex as a precursor to ribosome assembly (Steitz *et al.*, 1988), and it is probable that 5S rRNA moves into the nucleus in this form. 5S rRNA is imported into the nucleus by either active transport or facilitated diffusion, and accumulates in the nucleus by means of specific binding in the nucleus to non-soluble elements such as at the nucleolus. 5S rRNA has been shown to accumulate at the nucleolus by the binding of biotin-labelled antisense oligonucleotides to oocyte sections (Raška *et al.*, 1995), and by immunogold labelling (Raška *et al.*, 1990) and fluorescence microscopy (Steitz *et al.*, 1988) using human autoantibodies to 5S RNPs. Binding of 5S rRNA (as 5S rRNA-containing RNPs) to non-diffusible elements within the nucleus could result in the import process proceeding down the chemical activity gradient, and therefore by facilitated diffusion rather than active transport, especially as it is likely that 5S rRNA is stored on the cytoplasmic side of the nuclear membrane as 7S RNPs but is imported into the nucleus as 5S RNPs.

In Chapter Three and Four, further evidence is provided for the involvement of L5 in signalling release of 5S rRNA from cytoplasmic storage, for subsequent nuclear import, and the potential role for 5S rRNA-binding proteins in providing nuclear targeting and retention signals is discussed.

Chapter Three
A Role for Ribosomal Protein L5 in 5S rRNA Nuclear Transport

3.1 Introduction

3.1.1 The importance of RNA-protein interactions

Most, if not all, RNAs are likely to exist within cells in specific complexes with proteins termed ribonucleoprotein particles (RNPs). These interactions serve to stabilise the RNAs, maintain the molecules in a functional conformation, and target them to different cellular locales (reviewed in Wickens & Dahlberg, 1987; Dreyfuss *et al.*, 1988). Formation of RNPs is important in such essential processes as the production of mature message RNA (Lührmann *et al.*, 1990), as well as signal recognition particle-mediated protein transport to the endoplasmic reticulum (Walter & Lingappa, 1986), and in the production of the protein biosynthesis machines; ribosomes (Hill *et al.*, 1990). The interaction between RNA and protein allows for a great variety of structural conformations in these complexes, resulting in the masking and exposing of signals for specific sub-cellular localisation and creation of sites for molecular interaction that enable different functional activities to take place.

3.1.2 Nuclear transport of RNPs

Transport of RNPs in both directions across the nuclear membrane requires the presence of signals that designate the complexes for movement through the nuclear pore complex (see Section 1.5). These signals may reside in the nucleotide sequence or conformation of the RNA, the amino acid sequence or structure of the bound protein, or in a combination of elements of both RNA and protein. Many proteins have been shown to be associated with RNA nuclear transport, some of which had been previously characterised as part of a stable RNP, and others which may have more transient interactions with the RNA. Table 3.1 gives a summary of the studies to date on proteins proposed to be involved in RNP nuclear transport. This table does not include proteins of the nuclear pore complex that have been found to directly

Table 3.1 Proteins involved in RNP nuclear transport

RNA	Protein(s) involved in transport	Direction of transport	Reference
mRNA (general)	hnRNP A1 CBP80 & CBP20	export	Piñol-Roma & Dreyfuss, 1992 Dargemont & Kühn, 1992
U snRNAs	CBP80 & CBP20	export	Izaurralde *et al.*, 1995
tRNA	glyceraldehyde-3-phosphate dehydrogenase	export	Singh & Green, 1993
5S rRNA	TFIIIA L5?	export	Guddat *et al.*, 1991
HIV-1 mRNA	Rev	export	Fischer *et al.*, 1994
Histone mRNA	3′-end processing activity	export	Eckner *et al.*, 1991
SRP RNA	SRP9/SRP14	export	He *et al.*, 1994
Y RNAs	La, Ro60, Ro52	export	Simons *et al.*, 1994
U1, U2, U4 and U5 snRNAs	Sm proteins	import	Fischer *et al.*, 1993; 1994

interact with RNPs in transit, or the factors involved in the general transport process(such as Ran/TC4 and importin), but rather focuses on the more specific interactions of proteins that form part of stable RNPs.

Evidence for the role of particular proteins in RNP transport comes from studies that demonstrate the inhibition of the transport process in the absence of protein binding. For example, two tRNA mutants that are unable to bind glyceraldehyde-3-phosphate dehydrogenase are not exported from the nucleus (Singh & Green, 1993); signal recognition particle RNA (SRP RNA) that does not bind the proteins SRP9 and SRP14 is retained in the nucleus (He *et al.*, 1994); the U1, U2, U4 and U5 snRNAs

must be capable of binding Sm proteins to be competent for nuclear import (Fischer *et al.*, 1993; 1994b); and the nuclear export of 5S rRNA in oocytes is inhibited in mutants that cannot bind TFIIIA or L5 (Guddat *et al.*, 1990). However, it has also been proposed that studies using mutant RNAs are not purely testing the role of the specific protein in their transport, as the mutations could result in the generation of artifactual sites enabling the RNA to bind to nuclear retention factors (Boelens *et al.*, 1995).

The only proteins for which there is direct evidence of an essential role in RNP transport are HIV-1 Rev protein (Fischer *et al.*, 1994) and the cap binding protein CBP20 (Izaurralde *et al.*, 1995). The presence of purified recombinant Rev protein was found to be essential for the export of mRNA containing the Rev recognition site (the Rev-responsive element) from *Xenopus* oocyte nuclei (Fischer *et al.*, 1994). Evidence for a role for CBP20 in transport was provided by the inhibition of U snRNA export from *Xenopus* oocyte nuclei by antibodies raised against recombinant CBP20 (Izaurralde *et al.*, 1995). The actual role of the identified protein components in RNP nuclear transport is unclear. They may provide nuclear transport signals (either for export or import), or alter the conformation of the RNA or bound protein to activate a masked signal. These proteins may also release the RNP from retention in either the nuclear or cytoplasmic compartments, allowing the RNP to enter the nuclear transport pathway.

3.1.3 5S rRNA-protein interactions and nuclear transport

5S rRNA-protein interactions serve to regulate the activity and subcellular localisation of oocyte-type 5S rRNA throughout oogenesis (Guddat *et al.*, 1990; Allison *et al.*, 1991; 1993; 1995). Therefore, investigation into the nuclear transport of 5S rRNA in oocytes needs to take into account a variety of interactions, that depend on both the temporal and spatial availability of different binding proteins. As is the case for other RNPs, it is possible that the signals for either import or export of 5S rRNA are due to its interaction with different proteins. 5S rRNA-binding proteins could enable either cytoplasmic or nuclear accumulation by supplying functional nucleocytoplasmic transport signals, by the 'deactivation' of the transport signal due to concealment within the 5S rRNA-protein complex, or by the creation of a retention

site for binding to static components, thereby preventing entry of 5S rRNA into the nuclear transport pathway.

Binding of either TFIIIA or ribosomal protein L5 has been proposed to be a requirement for nuclear export of newly synthesised 5S rRNA in *Xenopus* oocytes (Guddat *et al.*, 1990), as described above. TFIIIA and L5 bind to a mutually exclusive, common binding site on 5S rRNA (Huber & Wool, 1986); therefore, the mutant 5S rRNA molecule may be inhibited in its nuclear export capability due to its failure to interact with only one of these proteins. It appears likely that TFIIIA is bound to 5S rRNA during its export out of the nucleus, as this transcription factor is involved with 5S rRNA both at its synthesis in the nucleus and during subsequent storage as a stable 7S RNP in the cytoplasm. However, 7S RNPs appear to be transported across the nuclear membrane in the direction of export only, as an immunohistochemical study detected these particles exclusively in the cytoplasm (Mattaj *et al.*, 1983), and a mutant of oocyte-type 5S rRNA which binds TFIIIA with greatly reduced affinity is still imported into the nucleus (Allison *et al.*, 1993). These observations suggest that, in addition to its role in 5S rRNA transcription, TFIIIA binding is required for 5S rRNA export and storage in the cytoplasm, but does not play a role in nuclear import at the onset of ribosome biogenesis.

After storage in the cytoplasm as 7S RNPs, 5S rRNA moves back into the nucleus for ribosome assembly. Given the apparent nuclear exclusion of 7S RNPs, the trigger for nuclear import must involve the release of TFIIIA from 5S rRNA, probably by the binding of another protein or proteins. The best candidate for this role is ribosomal protein L5, with which 5S rRNA forms 5S RNPs prior to ribosome assembly (Steitz *et al.*, 1988; Allison *et al.*, 1991; 1993). Maximal synthesis of L5 coincides with the synthesis of other ribosomal proteins and ribosome assembly in stage III oocytes (Wormington, 1989), whereas TFIIIA concentration is highest prior to stage III, with levels subsequently decreasing (Dixon & Ford, 1982; Ginsberg *et al.*, 1984). Note that the protein components of 42S storage RNPs are no longer detectable in vitellogenic oocytes (Dixon & Ford, 1982), therefore 5S rRNA does not bind in these complexes at the later stages in oogenesis when some 7S RNPs are still present. The differential synthesis of TFIIIA and L5 during oocyte development results in the storage of 5S rRNA as 7S RNPs earlier in development, and the

subsequent out-competing of the very stable TFIIIA interaction by L5 to coincide with 5S rRNA nuclear import for ribosome assembly (Allison *et al.*, 1991). Thus, the shift is made from 5S rRNA storage in the cytoplasm to incorporation with other ribosomal RNAs and proteins into the ribosome.

5S rRNA and L5 occur in equimolar ratios in ribosomes where they remain tightly bound as 5S RNPs that can be released intact from dissociated ribosomal subunits (Blobel, 1971). Therefore, it would seem advantageous if the nuclear import of these two essential ribosomal components occured simultaneously at the onset of ribosome biogenesis, rather than the molecules being imported by two separate pathways. However, the nuclear import of the U1 snRNA-binding protein U1A, which is essential to the assembly of active U1 snRNPs, is imported independently of U1 snRNA via a facilitated process through the nuclear pore complex (Kambach & Mattaj, 1992). U1A appears to shuttle between nucleus and cytoplasm, with the number of U1A binding sites (on U1 snRNA) on either side of the nuclear membrane resulting in the establishment of an equilibrium between the two compartments. The U1 snRNA-specific 70K protein (Romac *et al.*, 1994) and the U2 snRNP-specific B″ protein (Kambach & Mattaj, 1994) also enter the nucleus independently and associate with the RNAs in the nucleus. It is possible that a similar mechanism could be operating with 5S rRNA in oocytes to establish the nucleo-cytoplasmic distribution of ribosomal protein L5. In this scenario, 5S rRNA and L5 could move into the nucleus independently, with the amount of 5S rRNA, and therefore the number of L5 binding sites, on either side of the nuclear membrane limiting the nuclear accumulation of L5. In oocytes, a cytoplasmic pool of 5S RNPs is known to exist (Allison *et al.*, 1991), demonstrating the presence of a potential cytoplasmic retention mechanism for L5. However, this mechanism is unable to explain what provides the stimulus for the nuclear mobilisation of 5S rRNA, which otherwise could be attributed to L5 binding prior to import.

3.1.4 The study outlined in this chapter

In Chapter Two, evidence was presented that the nuclear import of oocyte-type 5S rRNA occurs via a facilitated, nuclear pore-associated process. The study outlined in this chapter investigated the nuclear transport of 5S RNPs, by microinjection of *in*

vitro-synthesised radiolabelled 5S RNPs into oocytes. These results demonstrate that pre-assembled 5S RNPs are stable in the oocyte and are imported into the nucleus via a facilitated, nuclear pore-associated process that can be inhibited by cytoplasmic ATP depletion, chilling, and wheat germ agglutinin. In addition, the nuclear import of microinjected oocyte-type 5S RNPs proceeded at a faster rate and to a greater extent than oocyte 5S rRNA alone, indicating that L5 binding may indeed be a prerequisite for 5S rRNA nuclear import.

3.2 Materials and Methods

3.2.1 *In vitro* synthesis of ^{35}S-5S RNPs

Xenopus oocyte-type 5S RNPs, labelled with ^{35}S-methionine, were synthesised from the L5 cDNA clone pSP6-L5b (Wormington, 1989; a kind gift from Dr W.M. Wormington, University of Virginia, Charlottesville, Virginia, USA). A rabbit reticulocyte lysate coupled transcription-translation system for templates with SP6 promoters (Promega, Madison, WI, USA) was programmed with 1 µg circular pSP6-L5b and 1 µg of *in vitro*-transcribed *Xenopus* oocyte-type 5S rRNA (synthesised as described in Section 4.2) in the presence of 40 µCi L-[^{35}S]-methionine (1 000 Ci/mmole, *in vivo* cell labelling grade; Amersham Australia Ltd. or DuPont NEN: Life Technologies Ltd., Auckland, New Zealand). The reaction mixture was set up according to the manufacturer's instructions and incubated for 2 h at 30°C. The ^{35}S-5S RNP mixture was stored at -80°C without further purification. Prior to loading into the microinjection needle, the ^{35}S-5S RNP mixture was centrifuged through a 0.22 µm filter (Ultrafree, Millipore, Bedford, MA, USA) to remove particulate matter and aggregates that tended to cause blockages in the needle.

The added 5S rRNA was judged to be in approximately 10 to 20 fold excess of the rabbit 5S rRNA in the lysate, as determined by agarose gel electrophoresis and ethidium bromide staining (at 1 mg/ml) of RNA extracted from the reticulocyte lysate, compared with known amounts of purified 5S rRNA. *Xenopus* 5S rRNA added in excess would be likely to compete the rabbit 5S rRNA for binding to newly-synthesised L5, thereby producing a population of oocyte-type 5S RNPs. To test for

the binding of *Xenopus* oocyte-type 5S rRNA during the translation process, ^{32}P-5S rRNA (synthesised as described in Section 2.2.1) was added to the translation reaction in the place of unlabelled oocyte-type 5S rRNA. The presence of a band of ^{32}P-5S rRNA in the same position as ^{35}S-L5 in a gel shift assay was determined as described below.

3.2.2 Microinjection and analysis of nuclear transport

Oocytes were removed from adult *Xenopus laevis* and prepared for microinjection as described in Section 2.2.2. Stage V-VI oocytes were injected with 50 nl of ^{35}S-5S RNP solution and incubated at 18°C in O-R2 containing 1 µg/ml cycloheximide (Sigma) to inhibit the synthesis of ^{35}S-labelled proteins in the oocytes from unincorporated ^{35}S-methionine remaining in the lysate solution. Cycloheximide does not interfere with protein import in oocytes (Kambach & Mattaj, 1992). Nuclei were dissected in ice-cold isolation medium (83 m*M* KCl, 17 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.4), and nuclear and cytoplasmic fractions were collected in microfuge tubes on ice (10 cytoplasms and nuclei per sample). Samples were prepared for protein electrophoresis by homogenisation in 250 µl isolation medium using a Gilson pipette tip, and the cellular debris was removed by centrifugation twice at 10 000 x *g* for 5 min at 4°C, with the cleared supernatant removed to a fresh tube after each spin. The proteins were precipitated with 5 volumes of acetone (1250 µl) at –20°C overnight and collected by centrifugation at 10 000 x *g* for 15 min. The dried pellets were dissolved in 60 µl of sample buffer (2% SDS, 100 m*M* dithiothreitol, 60 m*M* Tris-HCl, pH 6.8, 0.01% bromophenol blue) by vigorous shaking for 1 to 2 h at room temperature.

The protein samples in loading buffer were denatured by boiling for 10 min, and placed on ice before loading on a discontinuous 12% polyacrylamide/0.1% SDS gel (Laemmli, 1970), of 20 cm in length and 0.75 mm thickness. After electrophoresis for 3 to 4 h at 200 V, the gel was fixed in 25% isopropanol:10% acetic acid:3% glycerol for 30 min with constant agitation, followed by soaking in Amplify (Amersham) for 25 min. The gel was air dried overnight on a plastic gel drying frame with cellophane cover (Tut's Tomb, Idea Scientific Co., Minneapolis, MN, USA), then exposed to Hyperfilm-MP (Amersham) at –80°C. The intensity of bands

on the fluorographs was measured using a Zeineh analytical hand-held scanning densitometer and Biomed Image Analysis software (Advanced American Biotechnology).

In the experiments to compare the nuclear import of microinjected ^{32}P-5S rRNA and ^{35}S-5S RNPs, the nuclear import of ^{32}P-5S rRNA was assayed as described in Section 2.2. ATP depletion and wheat germ agglutinin (WGA) treatments were also performed as described in Section 2.2. 50 nl of ^{35}S-5S RNP solution was injected into the cytoplasm of each oocyte after preinjection of apyrase (grade VII, Sigma) or WGA (Sigma).

3.2.3 Electrophoretic mobility shift assay for 5S RNPs

To test for the binding of *in vitro*-synthesised ribosomal protein L5 to 5S rRNA, an electrophoretic mobility shift assay was performed, as described in Wormington (1989). Samples from the translation reaction were loaded onto an 8% polyacrylamide gel and electrophoresed in TBE at room temperature for 4 h at 100 V. After electrophoresis, the gel was fixed in 25% isopropanol:10% acetic acid for 30 min, and dried at 80°C for 2 h under vacuum, followed by autoradiography. Note that with the exception of the assay for ^{35}S-5S RNP stability after injection into oocytes, these RNP gels were not treated for fluorography.

To verify the binding of *Xenopus* oocyte-type ^{32}P-5S rRNA to *in vitro*-synthesised ^{35}S-5S RNPs, two films were placed over the fixed and dried gel, according to the method of Pippin *et al.* (1994), and exposed at −80°C. This enabled the visualisation of the RNA-protein interaction by the presence of ^{32}P-5S rRNA in the same position as ^{35}S-5S RNPs.

For the analysis of ^{35}S-5S RNP stability within oocytes, 50 nl of the ^{35}S-5S RNP mixture was cytoplasmically injected into oocytes. After overnight incubation, 10 oocytes were pooled, and homogenised in 30 µl RNP homogenisation buffer (20 m*M* Tris, pH 7.6, 100 m*M* KCl, 1.5 m*M* $MgCl_2$, 2 m*M* dithiothreitol, 0.1 m*M* phenylmethylsulphonyl fluoride, 10 U/ml RNasin [Promega]) using a Gilson pipette tip. Cellular debris (yolk and pigment) was separated from the samples by

centrifugation at 10 000 x *g* for 10 min at 4°C, and the cleared supernatant was removed to a new tube. Glycerol-dye loading buffer (20 mM EDTA, 5% glycerol, 0.025% bromophenol blue, 0.025% xylene cyanol) was added to the samples prior to loading on the denaturing gel. ^{32}P-5S rRNA was run on the gel as a marker. The gel was divided into ^{32}P and ^{35}S portions to enable separate processing of the gels to account for the different signal strengths between the two isotopes. The ^{32}P-containing gel portion was dried directly and the ^{35}S-portion fixed for 25 min in 25% isopropanol:10% acetic acid, and soaked in Amplify for a further 25 min prior to drying. The gel sections was exposed separately to Hyperfilm-MP at −80°C.

3.3 Results

3.3.1 *In vitro* synthesis and characterisation of ^{35}S-labelled *Xenopus* oocyte-type 5S rRNA-containing 5S RNPs

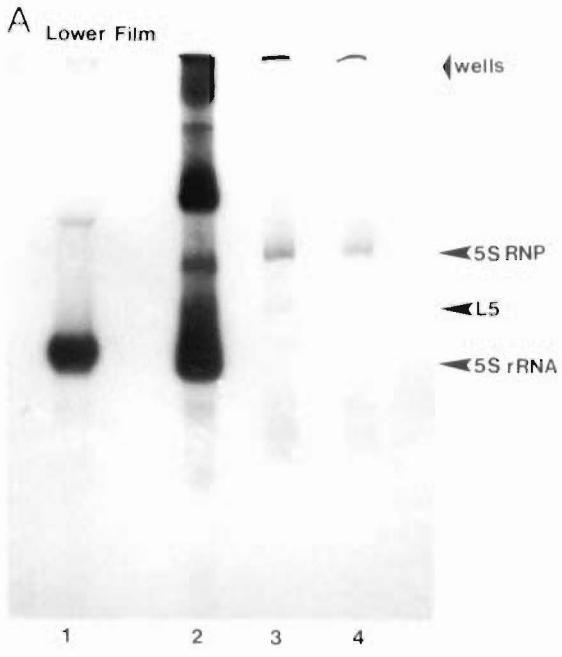
5S RNPs, with the ribosomal protein L5 component labelled with ^{35}S-methionine, were synthesised *in vitro* in a rabbit reticulocyte lysate-based coupled transcription/translation system. Initially, my aim was to produce ^{35}S-L5 to use in nuclear transport assays. However, non-denaturing gel electrophoresis of the post-translation reaction mixture revealed that most of the L5 produced was bound with rabbit 5S rRNA to produce 5S RNPs, as demonstrated by the slower migrating band indicated in Figure 3.1 A (lane 3) and 3.1 C (lane 2). This contrasts with the results of the study by Wormington (1989), where the *in vitro* translation of L5 in a wheat germ extract system resulted in only a small proportion (less than 25%) of the newly-translated L5 being bound as 5S RNPs. The rabbit 5S rRNA had the potential to interfere with an accurate comparison between the nuclear import of the 5S RNPs and oocyte-type 5S rRNA injected alone. Somatic-type and oocyte-type 5S rRNAs from *Xenopus* display differential associations with 5S rRNA binding proteins (Allison *et al.*, 1995), and mammalian 5S rRNA has been found to be more similar to *Xenopus* somatic-type 5S rRNA than oocyte-type (Ford & Southern, 1973). Therefore, it was important to either remove the rabbit 5S rRNA after translation, or provide excess oocyte-type 5S rRNA in the translation reaction mixture for the newly translated ^{35}S-L5 to bind to produce *Xenopus* oocyte-type 5S RNPs. Attempts to remove the rabbit

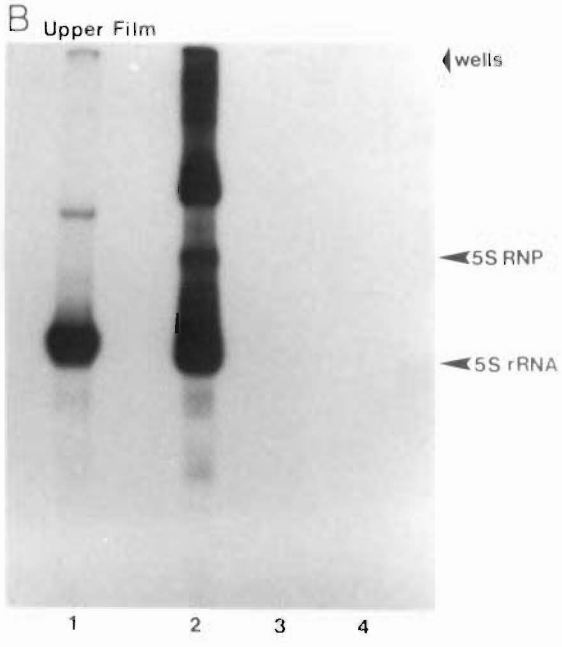
5S rRNA after translation by incubation with micrococcal nuclease (using the methods of Darby & Joho, 1992) were not successful, as the L5 was lost during this treatment (data not shown). This is probably due to the inherent instability or insolubility of L5 when not complexed with 5S rRNA (Huber & Wool, 1986; Yeh & Lee, 1995). An alternative method was therefore used, to result in the production of oocyte-type 5S RNPs.

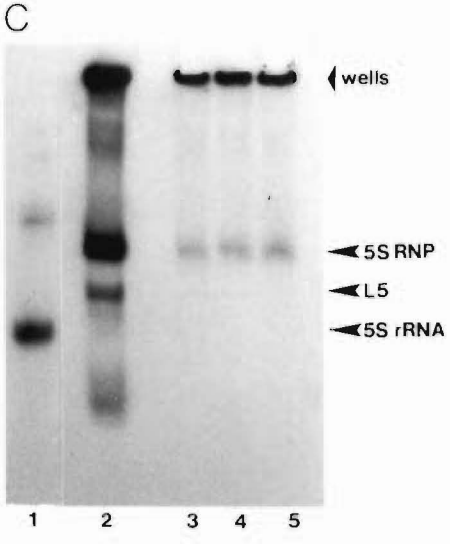
In vitro transcribed *Xenopus* oocyte-type 5S rRNA was added to the translation reaction mixture programmed with pSP6-L5b, in 10 to 20 fold excess of the rabbit 5S rRNA. The added 5S rRNA should presumably out-compete the rabbit 5S rRNA in the extract for binding to the newly translated L5, producing a population of oocyte-type 5S RNPs. While this study was in progress, a similar technique was published for the *in vitro* analysis of the binding of yeast ribosomal protein L1 to 5S rRNA (Yeh & Lee, 1995). To test whether the added oocyte-type 5S rRNA was binding to the newly transcribed L5, ^{32}P-labelled 5S rRNA was added to a translation reaction. A sample of the reaction mixture was run on a non-denaturing gel, with unbound ^{32}P-5S rRNA and ^{35}S-5S RNPs synthesised in the absence of ^{32}P-5S rRNA as size markers. The fixed and dried gel was overlaid with two X-ray films, according to the method of Pippin *et al.* (1994). ^{35}S has a lower energy emission than ^{32}P, and therefore the ^{35}S signal will only expose the bottom film, while ^{32}P can expose both films. Thus, the presence of ^{32}P-5S rRNA at the same position as the ^{35}S-5S RNPs on the film indicated the binding of added oocyte-type 5S rRNA to nascent L5.

As shown in Figure 3.1 A and B, ^{32}P-5S rRNA was detected in the translation reaction in the upper film at the same position as the ^{35}S-5S RNP band was detected in the lower film (cf. lanes 2, 3, and 4). This both verifies the binding of added 5S rRNA to newly synthesised L5, and unambiguously identifies the shifted band as the location of the 5S RNPs. *In vitro*-generated ^{35}S-5S RNPs microinjected in the oocyte cytoplasm remain stably bound as 5S RNPs in the oocyte after 18 hours of incubation as shown in Figure 3.1 C (cf. lanes 2 and 3-5), producing a band migrating more slowly than uninjected 5S rRNA (lane 1) and uninjected ^{35}S-L5 (lane 2). The 5S RNP band corresponds in mobility to the L5/5S rRNA complex formed by cytoplasmically microinjected ^{32}P-5S rRNA, as characterised in Allison *et al.* (1995). Note that the slower migrating band in the lane containing ^{32}P-5S rRNA (lane 1) only is not an

Figure 3.1: Characterisation of *in vitro*-synthesised ^{35}S-5S RNPs.
(**A**) and (**B**) Verification of binding of 5S rRNA to ^{35}S-labelled ribosomal protein L5 *in vitro*. ^{32}P-labelled oocyte-type 5S rRNA (lane 2), no 5S rRNA (lane 3), or unlabelled 5S rRNA (lane 4) was added to the ^{35}S-L5 translation reaction mixture. After non-denaturing gel electrophoresis, the gel was overlaid with two X-ray films. ^{32}P-5S rRNA alone was run in lane 1 as a marker. The locations of unbound 5S rRNA, unbound L5, and 5S rRNA complexed with L5 (5S RNP) are indicated. (**A**) Lower film, exposed by both ^{35}S and ^{32}P. (**B**) Upper film, exposed only by ^{32}P.
(**C**) Stability of ^{35}S-5S RNPs after microinjection into the cytoplasm of *Xenopus* oocytes. ^{35}S-5S RNPs were cytoplasmically injected into oocytes. After 18 h, the oocytes were homogenised and electrophoresed on an 8% polyacrylamide gel. Lane 1, ^{32}P-5S rRNA marker; lane 2, uninjected ^{35}S-5S RNP/^{35}S-L5 marker produced *in vitro* without the addition of *Xenopus* oocyte-type 5S rRNA; lanes 3 to 5, homogenates of ^{35}S-5S RNP-injected oocytes.


A
Lower Film
wells
5S RNP
L5
5S rRNA
1
2
3
4


B
Upper Film
wells
5S RNP
5S rRNA
1
2
3
4


C
wells
5S RNP
L5
5S rRNA
1
2
3
4
5

RNA-protein complex, but probably represents an artifact of the transcription process, where some of the 5S rRNA formed may be of a slightly different conformation or length. This band has been found to be reproducibly present on this type of gel (see also Allison *et al.*, 1995 and this thesis, Fig. 4.4 B). The higher molecular weight bands in lane 2 of Fig. 3.1 A, B and C probably correspond to non-specific complexes formed by interaction of ^{32}P-5S rRNA or ^{35}S-L5 with proteins of the reticulocyte lysate and the oocyte respectively.

3.3.2 Comparison of the nuclear import kinetics of microinjected ^{35}S-5S RNPs with ^{32}P-5S rRNA

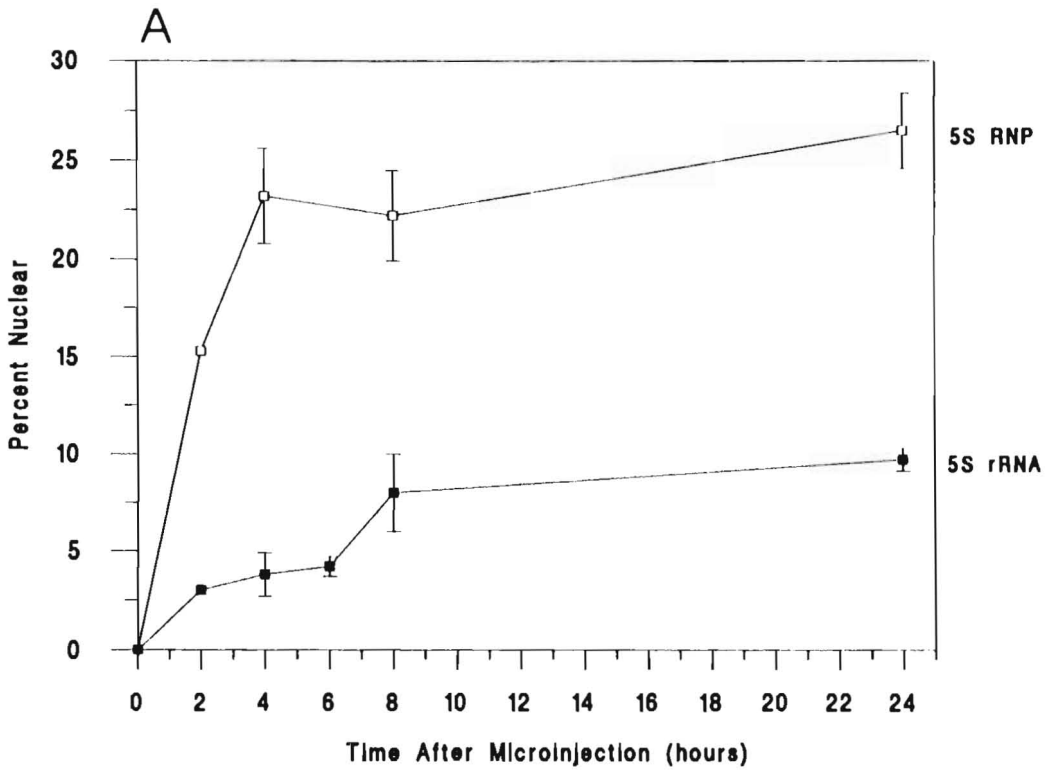
Having established a technique for generating stable ^{35}S-5S RNPs, I was then able to proceed to the analysis of their nuclear transport characteristics after microinjection into the oocyte cytoplasm. To assess whether the rate and extent of nuclear accumulation differ between 5S rRNA microinjected alone and the pre-assembled 5S RNPs, ^{32}P-5S rRNA and ^{35}S-5S RNP import with time was assessed in parallel. If there is a prerequisite of L5 binding to 5S rRNA for nuclear import to take place, then the import of the pre-assembled 5S RNPs should proceed at a faster initial rate than 5S rRNA injected alone, as there will be a lag time for the assembly of 5S rRNA into transport-competent 5S RNPs. As shown in Fig. 3.2 A, 5S RNPs were found to accumulate in the nucleus at a greater rate than 5S rRNA. The final net extent of import was also found to be greater for ^{35}S-5S RNPs, with a level of 25% nuclear accumulation after 24 hours compared with approximately 8% for ^{32}P-5S rRNA after this time. The same batch of oocytes was used for the nuclear transport assays for both ^{32}P-5S rRNA and ^{35}S-5S RNPs.

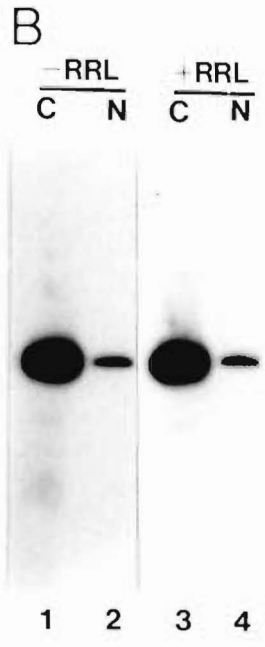
As the ^{35}S-5S RNPs microinjected in these assays for nuclear transport were not purified from the translation reaction mixture, it is possible that the rabbit reticulocyte lysate could be supplying factors that enhance nuclear transport (as suggested in Garcia-Bustos *et al.*, 1991b). This could bias the results in favour of the nuclear accumulation of ^{35}S-5S RNPs, when compared with ^{32}P-5S rRNA injected unbound. Therefore, the effect of the reticulocyte lysate on the nuclear transport of microinjected ^{32}P-5S rRNA was investigated. As shown in Fig. 3.2 B, the nuclear import of ^{32}P-5S rRNA mixed with the rabbit reticulocyte lysate proceeded at the same

Figure 3.2: Nuclear accumulation of cytoplasmically microinjected ^{32}P-5S rRNA and ^{35}S-5S RNPs with time.

(A) Quantitation of nuclear accumulation after microinjection into the cytoplasm. ^{32}P-5S rRNA or ^{35}S-5S RNPs (in rabbit reticulocyte lysate) were microinjected into the oocyte cytoplasm. At various time points as indicated, oocytes were manually dissected. RNA or protein was extracted from 5 or 10 pooled nuclear and cytoplasmic fractions, respectively, and separated by denaturing gel electrophoresis, followed by autoradiography (^{32}P-5S rRNA) or fluorography (^{35}S-5S RNPs). The percentage of radioactivity in the nucleus was quantified by densitometry. The bars indicate the standard error of the means from three sample replicates.

(B) ^{32}P-5S rRNA import is not affected by factors in the rabbit reticulocyte lysate. ^{32}P-5S rRNA was cytoplasmically microinjected into oocytes in a mixture either without (– RRL) or with (+ RRL) rabbit reticulocyte lysate. After 18 h incubation, nuclei were manually dissected and RNA was extracted from 5 pooled nuclear (N) and cytoplasmic (C) fractions. The samples were then separated by polyacrylamide gel electrophoresis as described above.


A
30
25
20
15
10
5
0
Percent Nuclear
0
2
4
6
8
10
12
14
16
18
20
22
24
Time After Microinjection (hours)
5S RNP
5S rRNA


B
-RRL
+RRL
C
N
C
N
1
2
3
4

rate and to the same extent as ^{32}P-5S rRNA injected without (cf. lanes 2 and 4). This figure shows an example of nuclear import after 24 hours. The same results were obtained when this analysis was performed at earlier time points (data not shown). Therefore, the rabbit reticulocyte lysate has no discernible effect on the nuclear transport process in these assays, and thus the enhanced rate of import must be due to the properties of the 5S RNP complex.

3.3.3 The effect of chilling on the nuclear import of ^{35}S-5S RNPs

As described in Chapter Two, chilling has been used as a criterion to establish whether nuclear transport of a molecule occurs via a facilitated or passive process. I have demonstrated that the nuclear import of microinjected ^{32}P-5S rRNA is reversibly inhibited by incubation at 0-4°C (Section 2.3.3). The effect of this treatment was tested on the nuclear import of microinjected ^{35}S-5S RNPs to see whether 5S RNP import also proceeds via this mechanism. As shown in Fig 3.3, the nuclear import of ^{35}S-5S RNPs was inhibited when the oocytes were incubated on ice at 0-4°C (cf. lanes 2 and 4). This effect was reversible by subsequent incubation at 18°C (lane 6), indicating that the chilling blocked RNP import by inhibiting the activity of specific components of the nuclear transport machinery, rather than preventing import by way of non-specific cellular damage. Note that the sample in lanes 4 and 6 was incubated for a longer time at 18°C (16 hours, after 6 hours chilled) than that shown in lanes 1 and 2 (6 hours); therefore, the greater amount of ^{35}S-5S RNPs detectable in the nuclear fraction is not the result of an enhancement in nuclear accumulation, but reflects the normal kinetics of 5S RNP import (as shown in Fig. 3.2). The results of this chilling study provide additional evidence for the binding of 5S rRNA and L5 during the import process, rather than an alternative and independent pathway for the nuclear accumulation of L5.

3.3.4 The effect of oocyte ATP depletion on the nuclear import of ^{35}S-5S RNPs

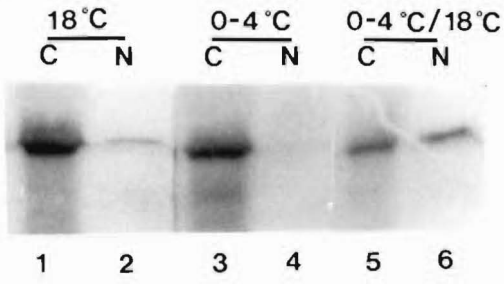
For a further characterisation of the nuclear import mechanism of microinjected ^{35}S-5S RNPs, the ATP-dependence of the process was tested. In the absence of ATP, energy-dependent facilitated nuclear transport is inhibited (Newmeyer & Forbes, 1988; Richardson *et al.*, 1988). Cytoplasmic ATP levels were depleted by microinjection of

Figure 3.3: The effect of chilling on the nuclear import of 5S RNPs.
Oocytes were injected with ^{35}S-5S RNPs and incubated at 18°C for 6 h (lanes 1 and 2), 0-4°C for 6 h (lanes 3 and 4), or 0-4°C for 6 h, then 18°C for 16 h (lanes 5 and 6). Nuclei were manually dissected and proteins were extracted by acetone precipitation from 10 pooled nuclear (N) and cytoplasmic (C) fractions. Samples were separated by 12% SDS-polyacrylamide gel electrophoresis, and the gel processed for fluorography.

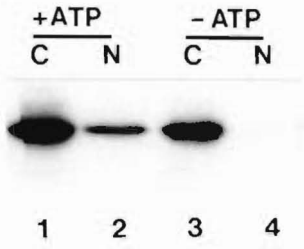
Figure 3.4: The effect of cellular ATP depletion on the nuclear import of 5S RNPs.
Oocytes were preinjected with phosphate buffered saline as a control (+ ATP) or with the ATP hydrolysing enzyme apyrase (– ATP), prior to cytoplasmic microinjection of ^{35}S-5S RNPs. After incubation at 18°C for 7.5 h, the assay for nuclear import was performed as described in the legend to Fig. 3.3. C, cytoplasmic fractions; N, nuclear fractions.

Figure 3.5: The effect of WGA on the nuclear import of 5S RNPs.
Oocytes were preinjected with phosphate buffered saline as a control (– WGA), WGA to a final cellular concentration of 2 mg/ml (+ WGA), or 2 mg/ml WGA and 50 mM *N*-acetylglucosamine (WGA + GlcNAc), prior to cytoplasmic microinjection of ^{35}S-5S RNPs. After incubation at 18°C for 18 h, the assay for nuclear import was performed as described in the legend to Fig. 3.3. C, cytoplasmic fractions; N, nuclear fractions.

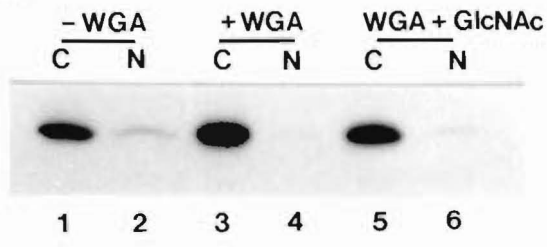
3·3


18°C
0-4°C
0-4°C/18°C
C N C N C N
1 2 3 4 5 6

3·4


+ATP
- ATP
C N C N
1 2 3 4

3·5


- WGA
+ WGA
WGA + GlcNAc
C N C N C N
1 2 3 4 5 6

the ATP hydrolysing enzyme apyrase (to a cellular concentration of 100 U/ml) as described in Section 2.3.4, which results in the rapid reduction of cellular ATP to 'background' levels. After this treatment, microinjected ^{35}S-5S RNPs were retained in the cytoplasm, as shown in Figure 3.4. Thus, as demonstrated for microinjected 5S rRNA, the process of 5S RNP import is ATP-dependent.

3.3.5 The effect of wheat germ agglutinin on the nuclear import of ^{35}S-5S RNPs

The final treatment used to verify that a facilitated mechanism is used in the nuclear import of 5S RNPs was the lectin wheat germ agglutinin (WGA). WGA inhibits facilitated nuclear transport by binding to *N*-acetylglucosamine residues on integral nuclear pore complex proteins, as described in Section 2.1.3. I have determined that the nuclear import of 5S rRNA microinjected alone is inhibited by pre-injection of WGA, with the amount of inhibition proportional to the concentration of WGA injected (Section 2.3.2). Likewise, the nuclear import of ^{35}S-5S RNPs was reduced in the presence of WGA (Fig. 3.5, cf. lanes 2 and 4). Co-injection of free *N*-acetylglucosamine, which binds to the WGA thereby reducing its binding to the nuclear pore proteins, reversed the inhibitory effect of the lectin (lane 6). The process of nuclear accumulation of ^{35}S-5S RNPs was less sensitive to WGA inhibition than ^{32}P-5S rRNA injected unbound, as a 20-fold higher concentration of WGA than that used with ^{32}P-5S rRNA was required for approximately the same degree of nuclear transport inhibition (cf. Fig. 2.2).

3.4 Discussion

In this study I have developed a technique for the *in vitro* synthesis of oocyte-type 5S rRNA-containing ^{35}S-labelled 5S RNPs, which can be used for microinjection assays in *Xenopus* oocytes. These 5S RNPs remain stable in the oocyte environment, and accumulate in the nucleus via a facilitated mechanism through the nuclear pore complex, which is inhibited by chilling, cytoplasmic ATP depletion, and WGA. This technique provides a useful method for the direct analysis of RNP nuclear transport

without relying on the assembly of the complex after microinjection of the naked RNA or protein.

3.4.1 Protein binding and the compartmentalisation of 5S rRNA

Movement of 5S rRNA within oocytes is dependent on protein binding. 5S rRNA appears to exit the nucleus bound with TFIIIA as 7S RNPs (Guddat *et al.*, 1990), and is retained in this complex in the cytoplasm awaiting a signal for nuclear import during ribosome biogenesis (Allison *et al.*, 1991). TFIIIA must possess a nuclear localisation signal (NLS) to enable the protein to enter the nucleus, where it functions as a transcription factor. Therefore, the question remains as to why TFIIIA bound as 7S RNPs with 5S rRNA is apparently unable to undergo nuclear import. There are two main possibilities that account for 7S RNP retention in the cytoplasm: either the nuclear targeting sequence is somehow masked or inactivated by the conformational change brought about by binding of 5S rRNA; or 7S RNPs bind to non-diffusible factors, such as the cytoskeleton, that prevent their entry into the nuclear transport pathway. However, isolated 7S RNPs are competent for nuclear import in some situations, as they are capable of accumulation into isolated *Xenopus* somatic cell nuclei in an *in vitro* transport assay (Allison, 1989), indicating that the binding of 5S rRNA may not affect the nuclear targeting signal. There is also evidence that 7S RNPs do not bind to elements of the cytoskeleton (M. North, pers. comm.). Therefore, another mechanism could be the concealment of the NLS within the complex formed by the dimerisation of 7S RNPs, as proposed in Callaci *et al.* (1990). This is likely to result in the inability of the NLS to associate with the nuclear transport machinery, as it must remain in an exposed position to be active (Roberts *et al.*, 1987). Masking of the NLS in 7S RNPs would ensure that 5S rRNA remains in storage in the cytoplasm, awaiting the signal for nuclear import and subsequent ribosome assembly. The results of this study provide evidence that the stimulus for 5S rRNA nuclear import may be due to the exchange of TFIIIA binding for ribosomal protein L5.

3.4.2 Comparison of 5S rRNA and 5S RNP nuclear import

The nuclear import of pre-assembled 5S RNPs microinjected into the oocyte cytoplasm proceeded at a faster rate and to a three-fold greater extent than 5S rRNA microinjected alone, suggesting that L5 binding is the first step in nuclear targeting. This result correlates with a previous study where the nuclear import of cytoplasmically injected oocyte-type and somatic-type 5S rRNAs was compared (Allison *et al.*, 1995). Somatic-type 5S rRNA had the same import profile as 5S RNPs, reflecting the fact that somatic-type 5S rRNA preferentially binds L5 *in vivo*, whereas oocyte-type preferentially binds TFIIIA (Allison *et al.*, 1995). The difference in the rate and extent of nuclear import between 5S rRNA injected alone and 5S RNPs could be due to the fact that the majority of injected 5S rRNA binds with TFIIIA and is retained in the cytoplasm, whereas the pre-assembled 5S RNPs are competent to enter the pathway of nuclear import. Therefore, the nuclear import of 5S rRNA injected alone is restricted by its limited ability to bind L5. The fact that the nuclear accumulation of 5S rRNA does not reach the levels of the pre-assembled 5S RNP after 24 hours, probably reflects the great capacity for this RNA to form storage 7S RNPs in preference over binding to L5.

Alternative interpretations can also be made for why there is greater nuclear accumulation of 5S RNPs when compared with 5S rRNA. It is possible that L5 is capable of nuclear import independent of binding to 5S rRNA, as must be the case in mammalian somatic cells where there is no significant cytoplasmic pool of 5S rRNA to which L5 could bind (Steitz *et al.*, 1988). In this case, 5S RNP formation occurs in the nucleoplasm, followed by targeting to the nucleolus. The L5 component of the microinjected 5S RNPs could therefore enter the nucleus by a different, more efficient pathway of nuclear import. However, this is not likely to be the case in these assays, as the microinjected 5S RNPs were found to remain intact within the oocyte.

Another explanation for the differential import profiles of 5S rRNA and 5S RNPs is that the nuclear accumulation of labelled 5S rRNA may reflect both the import of the RNA as 5S RNPs and its subsequent export bound to TFIIIA, in the same time frame that labelled L5 (either bound with 5S rRNA or not) is retained in the nucleus. It has been proposed that excess 5S rRNA in the nucleus may be

targeted to the cytoplasm of mammalian somatic cells for degradation bound to a TFIIIA-like protein (Lagaye *et al.*, 1988). Similarly, 5S rRNA present in the oocyte nucleus in excess of requirements for ribosome assembly may be exported for storage. Furthermore, the study of Allison *et al.* (1993) found that cytoplasmically injected labelled 5S rRNA does appear in 7S RNPs in the nucleus, indicating that imported 5S rRNA may form 7S RNPs. In this scenario, L5 would be retained in the nucleus even if the 5S rRNA with which it has been imported dissociates from it. Nuclear retention of L5 could occur by binding to elements such as ribosomal proteins, rRNAs or other components of the nucleolus, as appears to be the case for U3 snoRNA (Terns *et al.*, 1995). L5 could also be held in the nucleus by the lack of a positively acting nuclear export signal on the molecule. This model could account for part of the differential accumulation of 5S rRNA and 5S RNPs, but it is likely that this phenomenon can be largely explained at the initial steps of the nuclear import process, with oocyte-type 5S rRNA nuclear accumulation controlled by its preferential binding to TFIIIA.

3.4.3 Facilitated transport of 5S RNPs

As previously demonstrated for ^{32}P-5S rRNA (see Chapter Two), ^{35}S-labelled 5S RNP import is inhibited by cytoplasmic ATP depletion, chilling, and WGA. This supplies evidence that L5/5S RNP import occurs via a facilitated process through the nuclear pore complex. A requirement for metabolic energy has also been reported for the nuclear transport of other RNPs; such as, nuclear import of the U snRNPs (Michaud & Goldfarb, 1992; Fischer *et al.*, 1991) and the export of ribosomal subunits (Bataillé *et al.*, 1990).

The concentration of WGA required to maximally inhibit ^{35}S-5S RNP import was approximately 20-fold higher than that which was previously demonstrated to inhibit ^{32}P-5S rRNA import to the same extent (Chapter Two). This type of differential inhibition of import with WGA has been previously observed in comparisons between karyophiles (Fischer *et al.*, 1991; Michaud & Goldfarb, 1992; 1993; Marshallsay & Lührmann, 1994); however, the mechanism operating has yet to be elucidated. This differential sensitivity may appear contradictory to the proposal that 5S rRNA moves into the nucleus bound with L5 as 5S RNPs, as the WGA sensitivity of the import process should be the same whether it is the RNA or the

protein that is tagged during the assay. An explanation for the different concentrations of WGA required to inhibit nuclear import of 5S rRNA and 5S RNPs could be the presence of WGA-binding factors in the reticulocyte lysate component of the ^{35}S-5S RNP mixture. A pool of proteins that possess *O*-linked *N*-acetylglucosamine residues that are not associated with the NPC has been identified (Holt & Hart, 1986; Hart *et al.*, 1989). Therefore, these factors in the lysate could titrate out the WGA and make less available for binding at the oocyte nuclear pore complex; hence the requirement for a higher concentration to overcome this effect. It is also possible that a small proportion of L5 is dissociating from the microinjected 5S RNPs and entering the nucleus via a different, less WGA-sensitive pathway than 5S rRNA/5S RNPs. As previously discussed, L5 must have a mechanism of moving into the nucleus independent of binding to 5S rRNA in somatic cells. However, I think that this is unlikely to explain the situation in the assays described here, as *in vitro*-synthesised 5S RNPs remain stable after injection into oocytes.

3.4.4 Summary

Specific interactions of RNA molecules with proteins in RNPs play a vital role in the regulation of the nucleo-cytoplasmic distribution of RNA, including the nuclear transport of 5S rRNA in *Xenopus* oocytes. Binding to L5 appears to be a prerequisite for nuclear accumulation of 5S rRNA after its storage in the cytoplasm; thus, the ability of L5 to exchange for TFIIIA binding on the 5S rRNA molecule regulates in part the process of nuclear import. The results of this study further emphasise the specialised nature of oocyte-type 5S rRNA and the importance of its differential interaction with the proteins TFIIIA and L5 during oogenesis.

Chapter Four details further investigation into the role of L5 in 5S rRNA nuclear accumulation, and discusses whether L5 provides an active nuclear targeting signal for 5S rRNA.

Chapter Four
Comparison of the 5S rRNA Nuclear Transport Pathway with that of Other Molecules

4.1 Introduction

Nucleo-cytoplasmic transport of molecules and complexes can be described as occurring by different transport pathways, depending on their interaction with intermediates in the nuclear transport process. In this final part of my study on 5S rRNA nuclear import in *Xenopus* oocytes, a comparison has been made between the import pathway for this molecule and that of other molecules that undergo transport to the nucleus.

4.1.1 The saturability of nuclear transport processes

The facilitated nuclear import and export of proteins and RNAs/RNPs involves interactions with factors that are present in limiting amounts within the cytoplasm and nucleus (reviewed by Powers & Forbes, 1994; Adam, 1995; Simos & Hurt, 1995), as well as the proteins of the nuclear pore complex (NPC; reviewed by Forbes, 1992; Panté & Aebi, 1993; 1994; Fabre & Hurt, 1994; Hinshaw, 1994; Rout & Wente, 1994). Therefore, the process of nucleocytoplasmic transport is saturable when the sites for molecular interaction on these nuclear transport factors are exhausted. This saturation effect is demonstrated by the reduction in the rate of transport of the molecule concurrent with an increase in its cellular concentration. By this criterion, a saturable transport process has been demonstrated in *Xenopus* oocytes for the nuclear export of tRNA (Zasloff, 1983), 40S and 60S ribosomal subunits (Khanna-Gupta & Ware, 1989; Bataillé *et al.*, 1990), mRNA (Dargemont & Kühn, 1992), U1 snRNA (Jarmolowski *et al.*, 1994), and signal recognition particle RNA (He *et al.*, 1994), as well as for the nuclear import of karyophilic proteins (Goldfarb *et al.*, 1986).

Further analysis of mediated nuclear transport of proteins and RNAs/RNPs has identified the components of the transported molecule/complex responsible for the rate-limiting interactions. The association of the nuclear localisation signal (NLS)

with components of the nuclear transport machinery appears to be a limiting element in protein nuclear transport, as the presence of the prototypical NLS of the SV40 T antigen inhibits the nuclear import of labelled nuclear proteins (Michaud & Goldfarb, 1991; 1992; 1993). In addition, the rate of nuclear import of U1, U2, U4 and U5 snRNPs in *Xenopus* oocytes has been found to be reduced in the presence of the free trimethylguanosine cap dinucleotide (m_3GpppG), demonstrating that this process is saturable due to the interaction of the 5′ cap structure of the RNA with factors involved in nuclear import (Fischer *et al.*, 1991; Michaud & Goldfarb, 1992). The nuclear export of mRNA and U1, U2 and U5 snRNAs is also dependent on the structure at the 5′ end of the molecule, as the transport process is inhibited by excess monomethylguanosine cap (Dargemont & Kühn, 1992; Jarmolowski *et al.*, 1994).

The specific factors or receptors responsible for the limiting steps in nuclear transport are yet to be identified; however, many candidate molecules have recently been isolated (summarised in Section 1.5; and by Powers & Forbes, 1994; Simos & Hurt, 1995). The saturable component of the protein import pathway is likely to be part of the nuclear pore targeting complex, with which the protein must bind prior to its association with the NPC (Imamoto *et al.*, 1995a,b,c). A lack of available NLS receptors (of the importin-α/karyopherin-α-type; Adam & Gerace, 1991; Enenkel *et al.*, 1995; Görlich *et al.*, 1994; 1995a,b; Imamoto *et al.*, 1995b; Moroianu *et al.*, 1995a,b; Radu *et al.*, 1995a,b; Weis *et al.*, 1995) would prevent the karyophilic protein docking at the nuclear pore and prevent its subsequent interaction with the NPC and nuclear import.

The monomethylguanosine cap-dependence of U1, U2 and U5 snRNA nuclear export is probably mediated via the cap binding proteins CBP20 and CBP80 (Izaurralde *et al.*, 1995), and the trimethylguanosine cap requirement for U1, U2, U4 and U5 snRNA/RNP nuclear import in *Xenopus* oocytes is probably communicated through the binding of a protein analogous to CBP20 and CBP80. A soluble oocyte cytosolic factor, which could be a cap binding protein, has been found to be required for the trimethylguanosine cap-dependent import of U1 and U2 snRNPs (Marshallsay & Lührmann, 1994). CBP80 has been found to directly interact with the human NLS-receptor hSRP1α (Weis *et al.*, 1995), providing evidence that cap-binding proteins also require a receptor analogous to those required for protein import. However, this

NLS-receptor may be the product of a different member of the SRP1 gene family than that involved in the pathway of import or export taken by 'typical' karyophilic proteins (Adam, 1995; Weis *et al.*, 1995).

4.1.2 Comparisons of nuclear transport pathways for different molecules

The analysis of limiting components of nuclear transport pathways has been extended to provide a comparison between the mechanism of transport of different types of karyophiles. Inhibition of the nuclear transport of a labelled molecule in the presence of an excess of another unlabelled karyophile demonstrates the utilisation of at least one common nuclear transport intermediate. Microinjection assays of this type in *Xenopus* oocytes have resulted in the grouping of nuclear proteins and the snRNAs into three classes of nuclear import pathway (Fischer *et al.*, 1991; Michaud & Goldfarb, 1991, 1992), and have determined that the nuclear export of RNAs occurs via different pathways (Jarmolowski *et al.*, 1994). A summary of the nuclear transport competition studies to date is provided in Table 4.1.

Synthetic proteins containing the SV40 large T antigen NLS have been used as prototypical karyophilic proteins in comparative studies of nuclear import (Michaud & Goldfarb, 1991; 1992; 1993; van Zee *et al.*, 1993; Gulizia *et al.*, 1994). The presence of the T antigen NLS inhibits the nuclear import of *Xenopus* nucleoplasmin and a group of labelled *Xenopus* nuclear proteins (Michaud & Goldfarb, 1992; 1993), as well as the preintegration complex of HIV-1 in human cultured cells (Guilzia *et al.*, 1994; Stevenson, 1994), adenovirus E1A protein in cultured monkey cells (van Zee *et al.*, 1993), and *Xenopus* U6 snRNA (Michaud & Goldfarb, 1992). However, this signal does not compete the import of *Xenopus* U1 to U5 snRNAs, indicating that these RNAs are imported into the nucleus via an alternative pathway that involves association with different factors (Michaud & Goldfarb, 1991, 1992).

The saturable factors involved in the nucleocytoplasmic transport of RNAs/RNPs appear to be varied and specialised for particular RNA types. The import of U3 and U6 snRNAs/RNPs differ from U1, U2, U4 and U5 snRNAs/RNPs (Michaud & Goldfarb, 1992). U1, U2, U4 and U5 snRNA/RNP nuclear import can be competed by a free trimethylguanosine cap nucleotide, but not the SV40 T antigen NLS. U3

Table 4.1 Comparison of nuclear transport pathways by competition

Competitor	Competes the transport of:	Direction of transport	Reference
SV40 T antigen NLS	*Xenopus* nucleoplasmin and 'most' *Xenopus* nuclear proteins	import	Michaud & Goldfarb, 1993
	Xenopus U6 snRNP		Michaud & Goldfarb, 1991
	HIV-1 preintegration complex		Stevenson, 1994
	adenovirus E1A		van Zee *et al.*, 1993
m_3GpppN capping nucleotide	*Xenopus* U1, U2, U4 and U5 snRNPs (in oocytes)	import	Fischer *et al.*, 1991 Michaud & Goldfarb, 1992
tRNA	mRNA	export	Dargemont & Kühn, 1992 Jarmolowski *et al.*, 1994 Pokrywka & Goldfarb, 1995
m^7GpppG capping nucleotide	*Xenopus* U1, U2 and U5 snRNAs	export	Jarmolowski *et al.*, 1994
	mRNA		Dargemont & Kühn, 1992
HIV-1 Rev activation domain	5S rRNA U snRNAs	export	Fischer *et al.*, 1995

small nucleolar RNA (snoRNA) nuclear import is competed by neither the capping nucleotide nor the SV40 NLS, while the RNA polymerase III-transcribed U6 snRNA differs from the RNA polymerase II-transcribed U1 to U5 snRNAs in that its import is saturable in the presence of the protein NLS. Therefore, three distinct targeting pathways exist for the nuclear import of U1 to U6 snRNA.

Comparative analysis has also been performed for the nuclear export of tRNA, mRNA, U snRNA and rRNA (Dargemont & Kühn, 1992; Jarmolowski *et al.*, 1994; Pokrywka & Goldfarb, 1995). The export of mRNA, U snRNAs and 40S ribosomal subunits is mediated by specific factors; in that their transport processes are self-inhibited at saturating levels, but are not competed by the other RNAs (Jarmolowski *et al.*, 1994; Pokrywka & Goldfarb, 1995). However, tRNA appears to have general competitive effects on the nuclear export of all RNAs, suggesting that it shares some common steps in RNA export (Dargemont & Kühn, 1992; Jarmolowski *et al.*, 1994; Pokrywka & Goldfarb, 1995). In contrast, saturating levels of 40S ribosomal subunits are able to stimulate tRNA export (Pokrywka & Goldfarb, 1995). This result implies the existence of intranuclear RNA binding sites which can be exhausted by the binding of ribosomal subunits, thereby releasing tRNA for nuclear export.

The Rev protein of the HIV-1 virus, which is responsible for the nuclear export of viral pre-mRNAs and RNAs containing the Rev binding site, also possesses a domain that interacts with factors involved in nuclear export (Fischer *et al.*, 1995). Furthermore, the presence of this activation domain conjugated to the non-nuclear protein bovine serum albumin (BSA), strongly inhibited the export of both U snRNAs and 5S rRNA (Fischer *et al.*, 1995), which have previously been found to exit the nucleus via independent pathways (Jarmolowski *et al.*, 1994). The interpretation of these results is that both U snRNAs and 5S rRNA bind proteins that are functionally equivalent to Rev, that subsequently interact with the same nuclear export factor (Fischer *et al.*, 1995). It should also be noted that Jarmolowski *et al.* (1994) were unable to saturate the 5S rRNA export pathway in their experiments, and therefore the common factor(s) between U snRNA and 5S rRNA export may not have been detectable. Interestingly, TFIIIA, the protein likely to be involved in 5S rRNA export (Guddat *et al.*, 1990), possesses a sequence with high homology to the Rev activation domain which may be responsible for the interaction with the factor saturated by Rev (Fischer *et al.*, 1995).

4.1.3 Comparison of the pathway for 5S rRNA nuclear import with other molecules

The coordinated nuclear transport of different molecules required for the same or related processes, such as the proteins and RNAs that comprise ribosomes, could be achieved via the utilisation of the same pathway of molecular interactions for all the transported molecules. In terms of the present study, it was interesting to determine whether nuclear import of 5S rRNA/RNP, a nucleolar-targeted ribosomal component, utilises any of the previously identified pathways of nuclear transport: that in common with most nuclear proteins, or those more specific mechanisms of U1 snRNA or U3 snoRNA. The process of 5S RNP import could be via a protein-like mechanism, with L5 providing the dominant signal, or it could be imported by some type of small RNA/RNP mechanism, with the RNA potentially playing a role in the process.

One of the 'competitor' RNAs used in this study was U3 snoRNA, a nucleolar RNA which has a role in 18S-5.8S-28S rRNA processing (for review see: Maxwell & Fournier, 1995). In most cells, U3 snoRNA is synthesised in the nucleus by RNA polymerase II and moves to the nucleolus without any journey into the cytoplasm (Terns & Dahlberg, 1994; Terns *et al.*, 1995). However, U3 snoRNA does possess the means to enter the nucleus in oocytes; the NLS of U3 snoRNA is dictated largely by a structural motif at the 3′ terminal stem, and does not require cap trimethylation or binding of Sm proteins (Baserga *et al.*, 1992; Michaud & Goldfarb, 1992). These requirements for U3 snoRNA import suggest that a specific nuclear transport pathway has evolved for this molecule, that may be utilised at different stages of cell development when U3 snoRNA undergoes a journey into the cytoplasm with subsequent import back into the nucleus. For example, during the differentiation of rat myoblasts to myotubules, there is a shift in the cellular distribution of U3 snoRNA from exclusively nuclear to accumulation in the cytoplasm (Glibetic *et al.*, 1992), suggesting that U3 snoRNA may possess a means to re-enter the nucleus if required at a later stage of differentiation.

Because of the nucleolar nature of U3 snoRNA, and its association with 18S-5.8S-28S rRNA, it was of interest to determine whether the pathway of nuclear import for U3 snoRNA utilises saturable factors in common with 5S rRNA. Localisation of

proteins to the nucleolus is a process that does not appear to be dependent on specific nucleolar targeting sequences, but is related to nuclear import and subsequent binding to rRNA or rDNA at the nucleolus (Dang & Lee, 1989; Meßmer & Dreyer, 1993; Schmidt-Zachmann & Nigg, 1993). Therefore, it is possible that the nucleolar localisation of U3 snoRNA and 5S rRNA/5S RNPs may occur via the utilisation of the same nuclear transport pathway. Alternatively, 5S rRNA/5S RNP import may be coordinated with other ribosomal proteins by using the same karyophilic protein-type nuclear import factors or may involve interactions with the same intermediates as U1 snRNA, as has been demonstrated for its export from the nucleus (Fischer *et al.*, 1995).

4.2 Materials and Methods

4.2.1 Plasmids and proteins used in this study

The U1 snRNA gene template (Hamm *et al.*, 1987) was provided by Dr D.S. Goldfarb (University of Rochester, Rochester, New York, USA). For transcription, it was linearised with *Bam HI* (Boehringer Mannheim). The U3 snoRNA gene template (pXlU3A′; Savino *et al.*, 1992) was provided by Dr S. Gerbi (Brown University, Providence, Rhode Island, USA). The template used for transcription of U3 snoRNA was produced by M. Ezrokhi (Brown University), using PCR as described in Terns and Dahlberg (1994). The template contains the T7 promoter, and produces U3 snoRNA with five extra nucleotides at the 3′ end (3′-UUUUA), which enhances the *in vivo* stability of the transcript. P(lys)-BSA made from RNase-free BSA was prepared by K. Reynolds (University of Rochester), as described in Michaud and Goldfarb (1991).

4.2.2 High-yield synthesis of RNA

Synthesis of unlabelled 5S, U1 and U3 templates was performed *in vitro* using the Ampliscribe reaction kit (Epicentre Technologies, Madison, WI, USA) according to the manufacturer's instructions. Each reaction contained 1 µg DNA template, and was incubated for 2 h at 37°C. For the production of m^7G-capped RNA transcripts,

necessary for the stability of U1 snRNA and U3 snoRNA in the oocyte, $m^7G(5')ppp(5')G$ capping nucleotide (New England Biolabs, Beverly, MA, USA) was added to 3 mM, with a ten-fold reduction in the amount of GTP (0.75 mM). The monomethylguanosine cap on *in vitro*-synthesised U1 snRNA has been found to be efficiently hypermethylated and assembled into nuclear import-competent U1 snRNPs after microinjection into oocytes (Fischer & Lührmann, 1990).

RNAs were purified as described in Chapter Two (Section 2.2.1) for ^{32}P-labelled 5S rRNA, and quantified by spectrophotometry at 260 nm. The typical yield was 180 to 200 µg of RNA per reaction. To ensure that the transcripts were of the correct size and undegraded prior to injection, samples were visualised by electrophoresis on 8% polyacrylamide/8M urea gels followed by ethidium bromide staining. The RNAs were dissolved in TE (10m*M* Tris-HCl, pH 7.6, 1m*M* EDTA) at a concentration of 10 to 20 µg/µl and stored at −80°C. The RNAs were all soluble at such a high concentration due to their small size: 41 kD for 5S RNA (121 nt), 56 kD for U1 snRNA (165 nt), and 75 kD for U3 snoRNA (220 nt).

^{32}P-5S rRNA used in these assays was synthesised *in vitro* as described in Section 2.2. Capped ^{32}P-U3 snoRNA was synthesised from the U3 snoRNA template in the same manner in the presence of 400 µM capping nucleotide.

4.2.3 Preparation of RNA from oocytes and liquid scintillation counting

Microinjections and incubation of oocytes was performed as described in Section 2.2. After incubation for various times following co-injection of ^{32}P-5S rRNA and 'competitor', nuclei were manually dissected out of oocytes and RNA extracted from five pooled nuclear and cytoplasmic fractions as described in Section 2.2. To quantify sample radioactivity, the extracted RNA was dissolved in 50 µl TE, pH 7.6 and added to 2 ml of Biodegradable Counting Scintillant (BCS_i, Amersham) in 6 ml glass scintillation vials (Wheatman Scientific, Millville, NJ, USA) for liquid scintillation counting. Sample radioactivity was measured over 15 minutes in a Wallac 1410 liquid scintillation counter (Pharmacia) on the ^{32}P channel. Background was obtained by counting RNA extracted from uninjected oocytes or TE alone, with both giving the same measurements.

The formation of ^{32}P-5S rRNA-containing RNPs within the oocyte was verified by non-denaturing gel electrophoresis of oocyte homogenates. Oocytes were injected with 20 nl ^{32}P-5S rRNA. After incubation at 18°C for 18-26 h, five whole oocytes were homogenised in 30 µl RNP homogenisation buffer (see Section 3.2) using a Gilson pipette tip, and centrifuged at 10 000 x *g* for 10 min at 4°C. One tenth of the sample volume of glycerol dye loading buffer (see Section 3.2) was added to the clear supernatant, which was then loaded onto a 6% polyacrylamide gel containing 0.1% Triton X-100. The gel was run at room temperature in TBE with 0.1% Triton X-100 for 1.5 hours, dried for 2 h at 80°C under vacuum, and exposed to X-ray film at –80°C.

4.3 Results

4.3.1 The saturability of 5S rRNA nuclear transport

To establish whether 5S rRNA nuclear import is a saturable process that involves interactions with intermediates in the transport pathway, approximately 0.01 to 0.02 pmoles of ^{32}P-5S rRNA per oocyte was microinjected into the cytoplasm in the presence of 'excess' unlabelled 5S rRNA. The presence of saturating levels of 5S rRNA should reduce its rate of import, as detected by a reduction in ^{32}P-5S rRNA present in the nucleus compared with that injected in the absence of competitor. Indeed, this was found to be the case. As shown in Fig. 4.1, the rate of nuclear accumulation of ^{32}P-5S rRNA was markedly reduced in the presence of unlabelled 5S rRNA (+ 5S rRNA) at 7.5 pmoles per oocyte, with 3% nuclear after 24 hours in the presence of unlabelled 5S rRNA as opposed to approximately 8% with no competitor. Repetitions of this assay with variation in the amount of unlabelled 5S rRNA added established that consistent import competition occurred only at concentrations of 5 pmole or more per oocyte (resulting in an approximately 2500-fold excess over the ^{32}P-5S rRNA; data not shown). The results of this assay demonstrate that 5S rRNA nuclear transport is mediated by saturable transport intermediates.

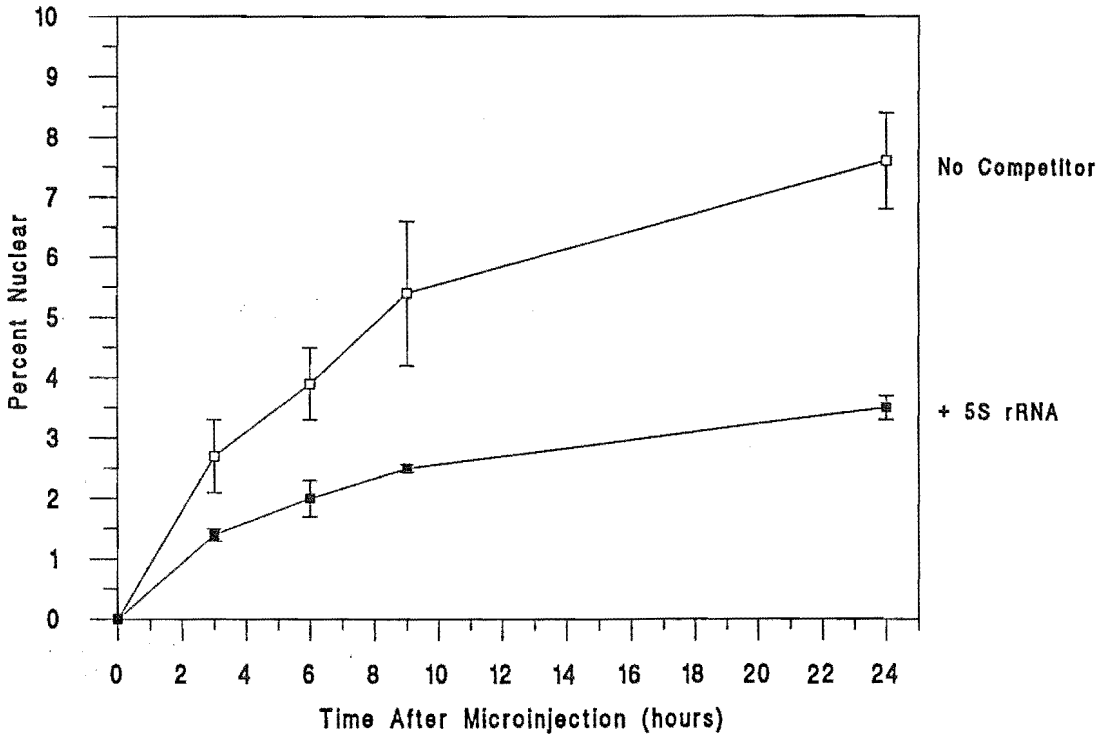


Figure 4.1: The saturability of 5S rRNA nuclear import.
^{32}P-5S rRNA was injected into the oocyte cytoplasm in the presence of unlabelled 5S rRNA at a concentration of 7.5 pmoles per oocyte (+ 5S rRNA), or with no unlabelled 5S rRNA (No Competitor). At various time points, oocytes were manually dissected and RNA was extracted from five pooled nuclear and cytoplasmic fractions for each sample. The percentage of radioactivity in the nucleus was quantified by liquid scintillation counting. Each point represents the mean from three sample replicates, with the bars indicating the standard error of the means.

Given this evidence for the presence of limiting factors in the 5S rRNA nuclear import pathway, it was of interest to determine whether other karyophilic molecules utilise the same components. For studies using heterologous RNAs, 5S rRNA self-competition was used as a positive control for the saturability of the transport process in all batches of oocytes, regardless of the rate and net extent of import. Note that ^{32}P-5S rRNA nuclear accumulation in the absence of competitor varied between batches, presumably due to slight differences in age and developmental stage (see also Fig. 2.1 for comparison). This variability has also been reported for the export of 5S rRNA after microinjection into oocyte nuclei (Fischer *et al.*, 1995).

4.3.2 The effect of U1 snRNA on 5S rRNA nuclear transport

The nuclear import of U1 snRNA/RNPs has been found to occur via a saturable process (Fischer *et al.*, 1991; Michaud & Goldfarb, 1991; 1992). To test whether 5S rRNA/5S RNPs utilise the same limiting component(s) during nuclear import as U1 snRNPs, U1 snRNA was microinjected with ^{32}P-5S rRNA at the same concentration (7.5 pmoles per oocyte) as that used for the self-competition experiments. The presence of 'excess' unlabelled U1 snRNA had no effect on the rate of nuclear accumulation of ^{32}P-5S rRNA, as shown in Fig. 4.2 (+ U1 RNA), demonstrating that U1 snRNA and 5S rRNA/5S RNPs are imported into the nucleus via different transport pathways.

4.3.3 The effect of U3 snoRNA on 5S rRNA nuclear transport

Although U3 snoRNA moves into the oocyte nucleus after cytoplasmic microinjection (Baserga *et al.*, 1992), it does not 'naturally' appear to undergo nuclear import in oocytes (Terns & Dahlberg, 1994; Terns, 1995). Therefore, it was important in these assays to assess whether cytoplasmically microinjected U3 snoRNA was being imported by a mediated process that would indicate a specific nuclear transport pathway. That is, U3 snoRNA must be able to compete its own import for the ^{32}P-5S rRNA competition assay to be valid. ^{32}P-U3 snoRNA was microinjected into oocytes in the presence and absence of 'competitor' unlabelled U3 snoRNA (at 7.5 pmoles per oocyte) and nuclear accumulation was measured over time. As shown in Fig. 4.3A, ^{32}P-U3 snoRNA nuclear transport was reduced in the presence of U3 snoRNA, with

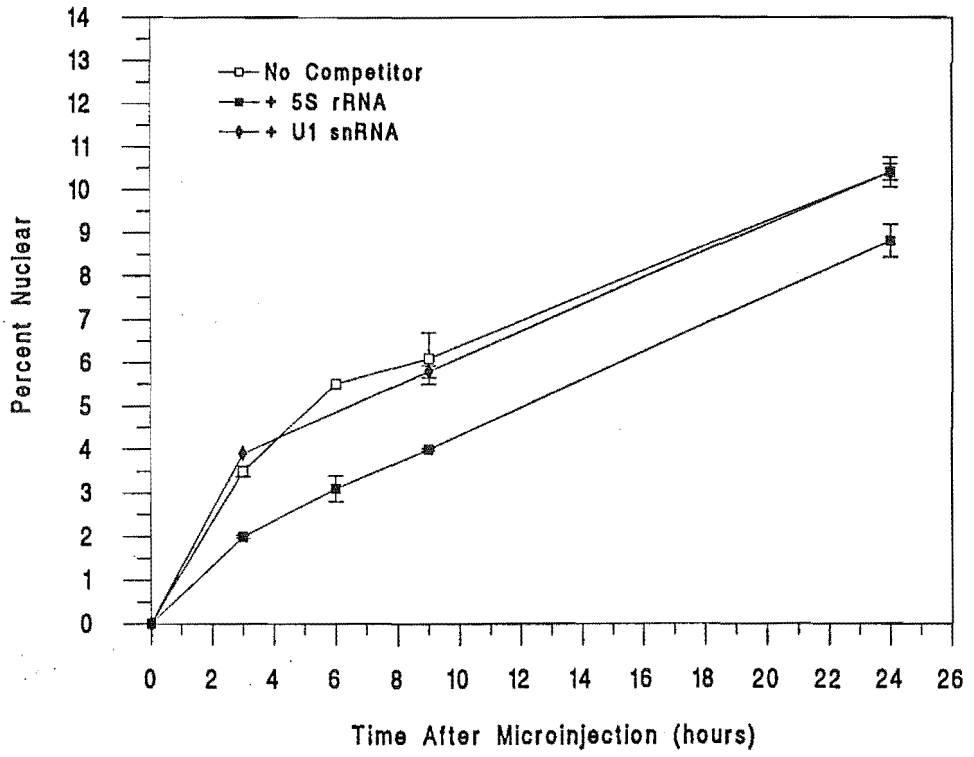


Figure 4.2: 5S rRNA import in the presence of U1 snRNA.
^{32}P-5S rRNA was injected into the oocyte cytoplasm with unlabelled U1 snRNA at a concentration of 7.5 pmoles per oocyte (+ U1 snRNA), with unlabelled 5S rRNA (+ 5S rRNA) or no competing RNA (No Competitor) as controls. The assay for nuclear import was performed as described in the legend to Fig. 4.1. The bars indicate the standard error of the means for three sample replicates.

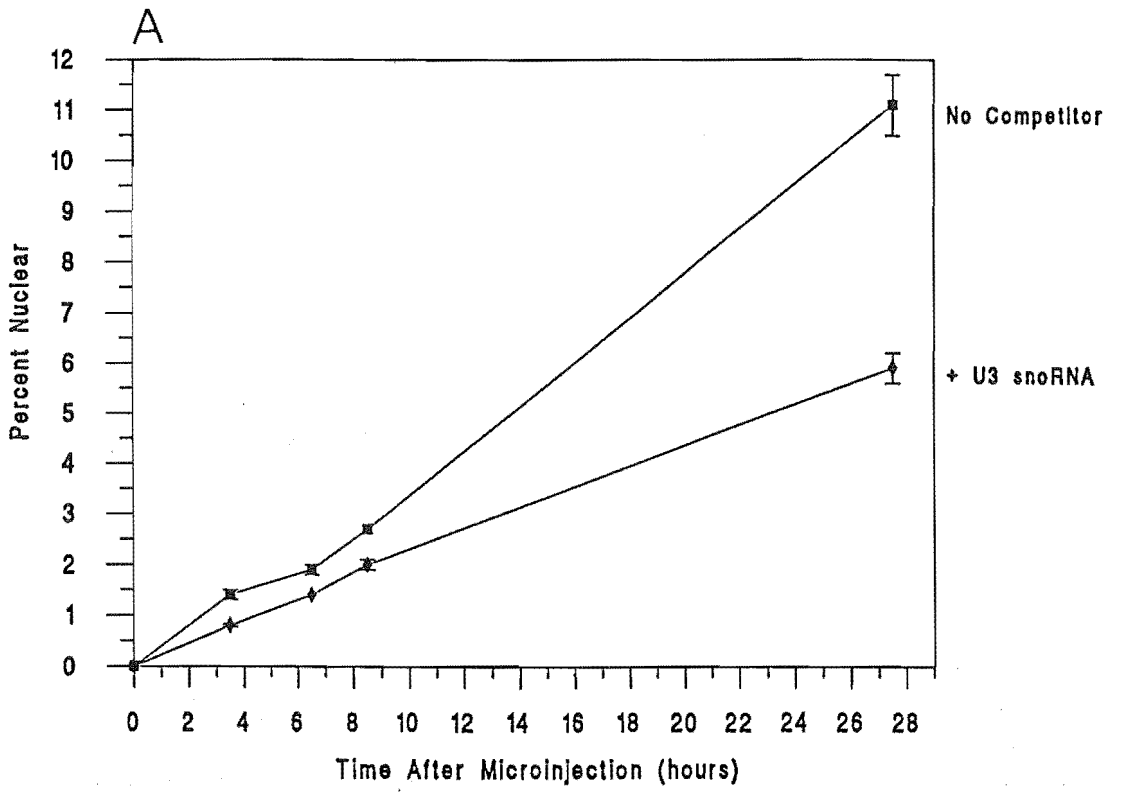
6% nuclear after 27 hours (+ U3 snoRNA) as opposed to 11% in the absence of competitor (No Competitor). Therefore, the nuclear import of U3 snoRNA occurs via a saturable, mediated process.

To test whether 5S rRNA nuclear import utilises the same limiting factors in its nuclear transport pathway as U3 snoRNA, ^{32}P-5S rRNA import was assayed in the presence and absence of U3 snoRNA. The presence of U3 snoRNA at 7.5 pmoles per oocyte had no effect on the nuclear accumulation of ^{32}P-5S rRNA, as shown in Fig. 4.3B. Therefore, 5S rRNA/5S RNP nuclear import does not proceed via the same saturable factors as this other nucleolar molecule.

4.3.4 The effect of the SV40 large T antigen nuclear localisation signal on 5S rRNA nuclear transport

The final treatment used in this comparative study of nuclear import was P(lys)-BSA. P(lys)-BSA is a synthetic protein formed by the conjugation of multiple copies of the minimal SV40 T antigen NLS to BSA (Michaud & Goldfarb, 1991). The use of this molecule enabled a comparison to be made between the pathway of nuclear import used by most nuclear proteins, and that of 5S rRNA. In contrast to the results with U1 snRNA and U3 snoRNA, the rate of nuclear import of ^{32}P-5S rRNA in the presence of 15 μM P(lys)-BSA was significantly reduced when compared with import in the presence of BSA to the same concentration (Fig. 4.4A). 5S rRNA nuclear accumulation was reduced from 16% when coinjected with BSA only, to 2% in the presence of P(lys)-BSA. P(lys)-BSA has the potential to alter RNA-protein interactions, thus inhibiting RNP formation (Michaud & Goldfarb, 1992). Inhibition of 5S rRNA binding to L5 could account for a reduction in import, by preventing the formation of the putative transport competent form of 5S rRNA. Therefore, the effect of P(lys)-BSA on the formation of 5S RNPs within the oocyte was tested. As shown in Fig. 4.4B, the extent of 5S RNP (and 7S RNP) formation was the same regardless of the concentration of P(lys)-BSA within the oocyte.

Figure 4.3: 5S rRNA import in the presence of U3 snoRNA.
(A) The saturability of ^{32}P-U3 snoRNA nuclear import. ^{32}P-U3 snoRNA was microinjected into the oocyte cytoplasm in the presence of unlabelled U3 snoRNA to a final concentration of 7.5 pmoles per oocyte (+ U3 snoRNA), or with no unlabelled (No Competitor). At various time points as indicated, RNA was extracted from five pooled nuclear and cytoplasmic fractions, and the percentage of radioactivity in the nucleus quantified by liquid scintillation counting. The bars indicate the standard error of the means from three replicate samples.
(B) Quantitation of nuclear accumulation of 5S rRNA in the presence of U3 snoRNA. ^{32}P-5S rRNA was microinjected into the oocyte cytoplasm in the presence of unlabelled U3 snoRNA to a final concentration of 7.5 pmole per oocyte (+ U3 snoRNA), or with 5S rRNA (+ 5S rRNA) or no added unlabelled RNA (No Competitor) for comparison. Nuclear accumulation with time was analysed as described in (A).


A
Percent Nuclear
Time After Microinjection (hours)
No Competitor
+ U3 snoRNA

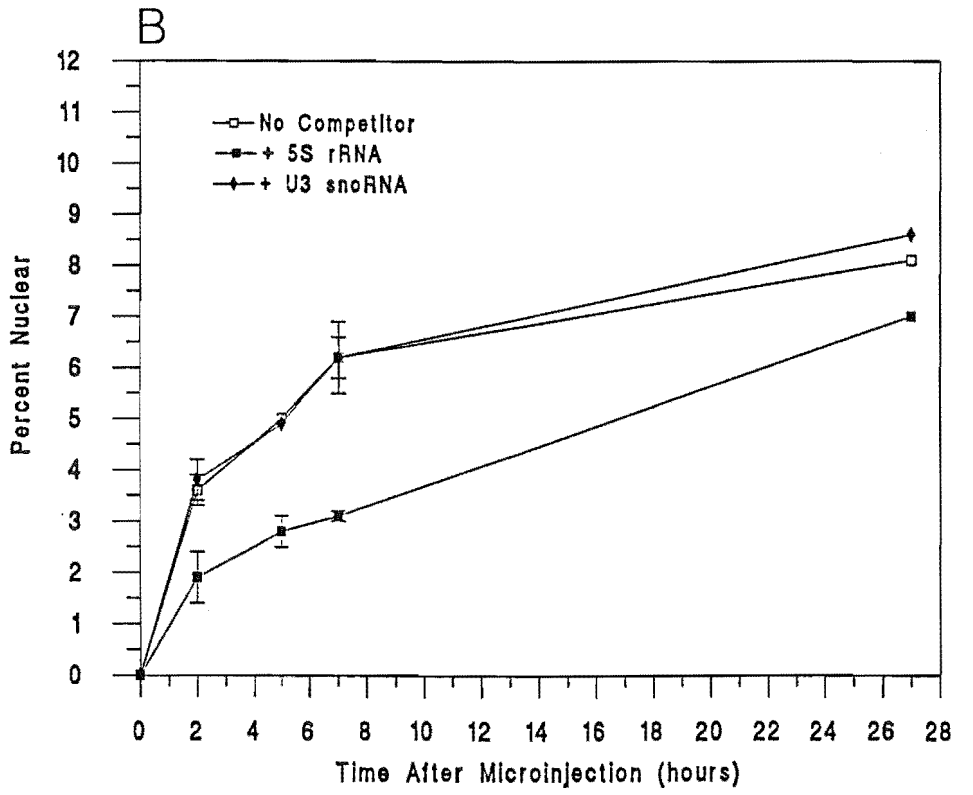
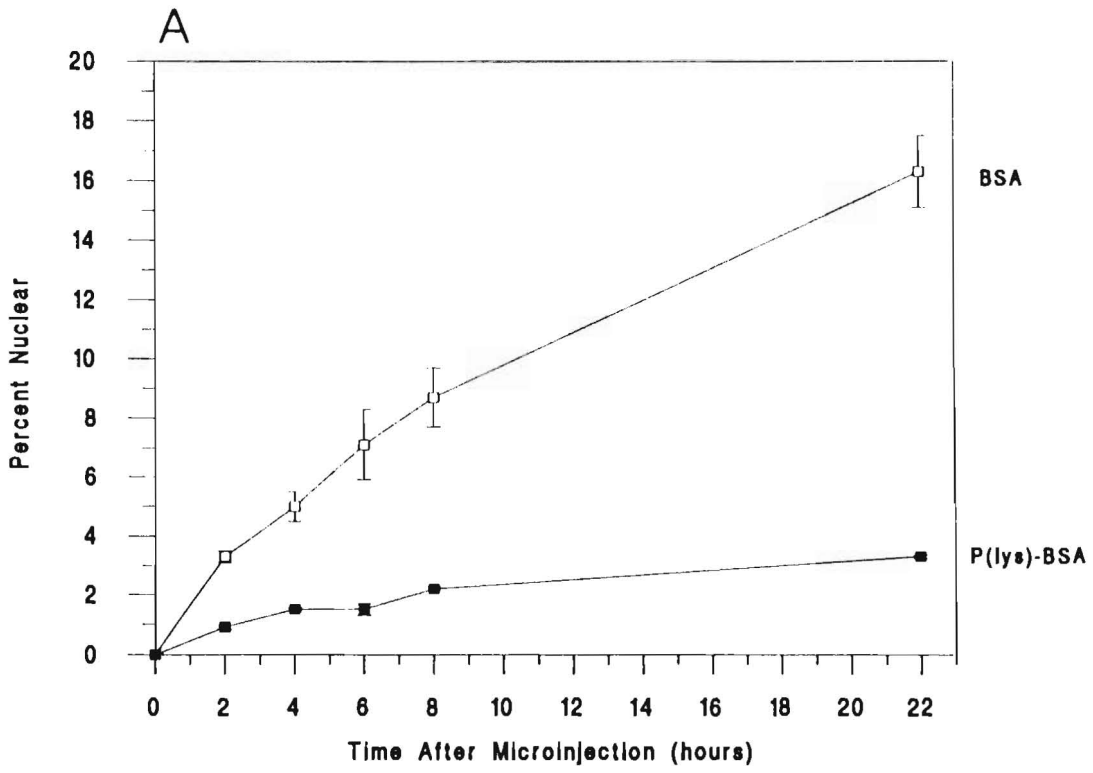
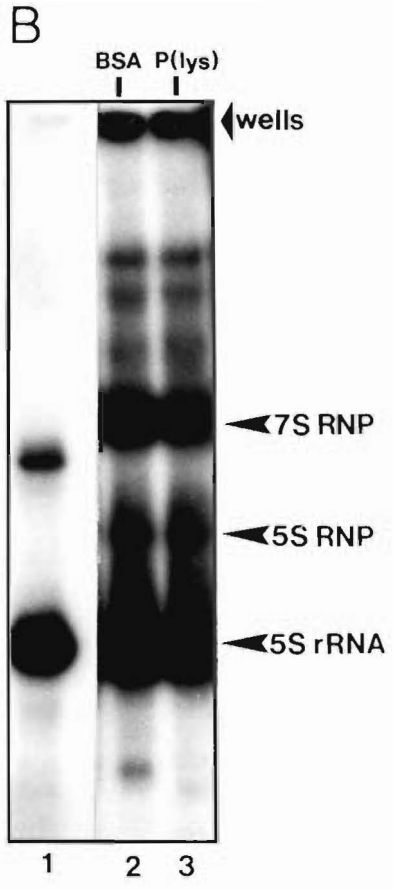

B
Percent Nuclear
Time After Microinjection (hours)
No Competitor
+ 5S rRNA
+ U3 snoRNA

Figure 4.4: 5S rRNA import in the presence of P(lys)-BSA.
(A) Quantitation of nuclear accumulation in the presence of P(lys)-BSA. ^{32}P-5S rRNA was microinjected into the oocyte cytoplasm with P(lys)-BSA (the minimal SV40 T antigen NLS coupled to bovine serum albumin) to a final cellular concentration of 15 μ*M*, or BSA (at the same concentration) as a control. Samples were analysed as described in the legend to Fig. 4.3.
(B) Formation of ^{32}P-5S rRNA-containing RNPs in the presence of P(lys)-BSA. ^{32}P-5S rRNA was injected into the oocyte cytoplasm in the presence of 15 μ*M* BSA (lane 2) or P(lys)-BSA (lane 3). After incubation at 18°C for 18 h, oocytes were homogenised and run on a non-denaturing 6% polyacrylamide gel containing 0.1% Triton X-100. A marker of uninjected ^{32}P-5S rRNA is shown in lane 1. The band migrating more slowly than 5S rRNA in lane 1 probably reflects an artifact of the transcription process, resulting in an RNA of different conformation or length. This band is reproducibly present on non-denaturing gels (see Fig. 3.1 and Allison *et al.*, 1993).


A
Percent Nuclear
20
18
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
14
16
18
20
22
Time After Microinjection (hours)
BSA
P(lys)-BSA


B
BSA
P(lys)
wells
7S RNP
5S RNP
5S rRNA
1
2
3

4.4 Discussion

The results presented in this chapter demonstrate that the pathway of nuclear import of 5S rRNA/5S RNPs involves interactions with receptors that can also bind karyophilic proteins, but does not have saturable components in common with either U1 snRNA or U3 snoRNA. Therefore, unlike these other RNPs, 5S rRNA/5S RNP nuclear transport follows a pathway similar to most nuclear proteins. The nuclear import of two other RNPs, the HIV-1 preintegration complex in growth-arrested cells (Stevenson, 1994), and U6 snRNP in *Xenopus* oocytes (Michaud & Goldfarb, 1992), is also competed by the SV40 T-antigen NLS.

4.4.1 Requirements for saturation of the 5S rRNA/5S RNP import pathway

In these experiments, saturation of the 5S rRNA nuclear import pathway was only achieved with a high concentration of competitor 5S rRNA (7.5 pmoles per oocyte compared to approximately 0.002 pmoles of ^{32}P-5S rRNA). In a previous study, the process of 5S rRNA nuclear export was not able to be inhibited by up to 5 pmole of competitor 5S rRNA per oocyte, whereas microinjection of 2.5 pmole of unlabelled U1 snRNA per oocyte was sufficient to completely suppress the export of labelled U1 snRNA (Jarmolowski *et al.* 1994). The nuclear export of labelled mRNA and tRNA is also self-inhibited in the presence of competitor at 5 pmole or less (Jarmolowski *et al.*, 1994). The difficulty in saturating 5S rRNA nuclear transport can be explained by the potential of microinjected oocyte-type 5S rRNA to form associations with either TFIIIA or L5, which signal either nuclear or cytoplasmic localisation.

Oocyte-type 5S rRNA differs from the other RNAs for which nuclear transport pathways have been studied, in that its distribution between nucleus and cytoplasm is the result of two processes: cytoplasmic storage and nuclear accumulation for ribosome assembly. Thus, 5S rRNA microinjected into either the nuclear or cytoplasmic compartments of oocytes can be involved in interactions leading to either of these two situations. It is possible in the case of nuclear import that most of the 5S rRNA injected is forming storage 7S RNPs in the cytoplasm, and is consequently unable to undergo nuclear import (Allison *et al.*, 1995); therefore, a high amount of

competitor oocyte-type 5S rRNA is required to produce enough 5S RNPs to bind to nuclear transport receptors and reduce the rate of nuclear accumulation. This scenario can also be related to the export process, with a requirement for enough export-competent RNPs (7S RNPs) to be formed before there can be any competition effect. Furthermore, some of the 5S rRNA in the nucleus is being assembled into 60S subunits at the nucleolus prior to export, and this would also reduce the potential for interaction with the nuclear export machinery.

It is also possible that 5S rRNA import and export occur via pathways that are more general than those utilised by other RNAs. A general pathway would presumably have transport intermediates at a higher concentration within the cell. If this is the case, it would take a greater amount of 5S rRNA to saturate these binding sites than for the other, more specialised, RNA pathways which may have fewer transport factors available. This scenario is supported by the evidence presented here that 5S rRNA import is competed by a proto-typical protein NLS, which indicates that the import process may occur by the general protein pathway. This pathway is responsible for the nuclear accumulation of a large variety of nuclear proteins (see Table 4.1) and would therefore require many copies of the transport factors. It should be noted that the amount of competitor that has been used in this and other studies to saturate protein import is at a higher molar concentration (15 to 25 μM; Michaud & Goldfarb, 1991; 1992; 1993) than the competitor RNAs, which completely inhibited nuclear export at concentrations of less than 10 μM (assuming an oocyte volume of 0.5 μl; Jarmolowski *et al.*, 1994). Furthermore, each molecule of P(lys)-BSA contains multiple copies of the NLS (Breeuwer & Goldfarb, 1990) and could possibly bind many transport factors.

4.4.2 Signals and receptors required for 5S rRNA/5S RNP nuclear import

Facilitated transport is mediated by nuclear localisation signals, therefore the question still remains as to the location of the NLS on the 5S RNP. The signal for the nuclear transport of RNPs could reside in the nucleotide sequence of the RNA, or it may be a component of the amino acid sequence of the bound protein (or proteins), or formed by a combination of sequences and conformation brought about by the RNA-protein interaction. Mutational analysis of 5S rRNA has shown that specific

structural elements of the 5S rRNA molecule are important for nuclear localisation, but no single region of 5S rRNA is solely responsible, suggesting that molecular shape, rather than specific sequence, plays a primary role in transport (Allison *et al.*, 1993; Allison & Romaniuk, in prep.). Therefore, the key to nuclear import may lie in the ability of the RNA to form a conformation that allows L5 to bind. As 5S rRNA/RNP nuclear transport was competed by a protein NLS, it is likely that the 5S RNP contains a similar targeting signal to this class of karyophiles. Since the sequence of L5 does possess a putative NLS (KKPKKFVKKK; Wormington, 1989), this signal probably resides on the protein component of the 5S RNP.

The inhibitory effect of P(lys)-BSA on 5S rRNA/RNP nuclear import may be due to competition for the same NLS receptor that targets both molecules to the NPC (reviewed by Simos & Hurt, 1995). This protein is likely to be the α subunit of *Xenopus* importin (Görlich *et al.*, 1994; 1995a,b), which is homologous to the human NLS receptor hSRP1α (Görlich *et al.*, 1995a; Weis *et al.*, 1995). Binding of a labelled SV40 T antigen NLS-protein conjugate to hSRP1α was found to be blocked in the presence of excess SV40 NLS (or the bipartite NLS of CBP80), demonstrating the saturability of this component of the nuclear transport machinery (Weis *et al.*, 1995). Failure of interaction of the NLS with its receptor would result in 5S RNPs being retained in the cytoplasm due to their inability to combine into the nuclear pore targeting complex and enter the nuclear import pathway (Görlich *et al.*, 1995a,b; Imamoto *et al.*, 1995a,b,c).

4.4.3 A role for shuttling proteins in 5S rRNA/5S RNP import?

The competition between P(lys)-BSA and 5S rRNA/RNP may also be for binding at an earlier stage in nuclear transport, to another molecule that subsequently interacts with the nuclear pore targeting complex. The nucleolar proteins nucleolin, No38/B23, and Nopp140 have been shown to shuttle across the nuclear membrane in both directions, suggesting the possibility that these proteins are involved in the ferrying of other karyophiles into the nucleus (Borer *et al.*, 1989). Furthermore, NLS-binding activity has been observed for some nucleolar proteins (Lee *et al.*, 1991; Meier & Blobel, 1990; 1992; Xue *et al.*, 1993), providing evidence for a role in nuclear transport. It is possible that 5S RNPs are imported into the nucleus, and

subsequently targeted to the nucleolus, bound with one of these nucleolar proteins. The fact that nucleolin is capable of binding the SV40 T-antigen NLS (Xue *et al.*, 1993), and that P(lys)-BSA is localised to the nucleolus after its nuclear import (Breeuwer & Goldfarb, 1990), suggests that P(lys)-BSA could associate with this shuttling protein in oocytes. This saturation of the NLS binding sites of the shuttling protein would prevent interaction with the NLS of 5S RNPs. Nucleolin has its own bipartite NLS (Schmidt-Zachmann & Nigg, 1993), which presumably must associate with the NLS receptor of the nuclear pore targeting complex prior to nuclear import. It is possible that the SV40 NLS on P(lys)-BSA is binding to both sets of NLS receptors, and doubly affecting 5S RNP nuclear import.

Binding to shuttling proteins may be a general phenomenon in the nuclear import of ribosomal proteins. In the case of 5S RNP nuclear import, it appears that 5S rRNA is simply being 'piggy-backed' by ribosomal protein L5, via a nuclear transport pathway that may be utilised by other ribosomal proteins, as well as L5 when it is not bound with 5S rRNA (in somatic cells which do not possess a cytoplasmic pool of 5S rRNA). SV40 T antigen or histone H2B NLSs alone conjugated with small, non-nuclear proteins were found to interact with the amino terminus NLS receptor domain of nucleolin (Xue *et al.*, 1993). No38/B23 has also been found to bind simple peptides containing the SV40 or HIV-1 Rev NLSs (Szebeni *et al.*, 1995), and Nopp140 binds the SV40 NLS cross-linked with human serum albumin (Meier & Blobel, 1990). This suggests that the requirement for interaction of the transport substrate with the shuttling protein may be contained solely within the NLS. However, there may be moderators of NLS binding for nuclear proteins *in vivo*. It is possible that the nuclear import of proteins destined for the nucleolus is achieved by a 'default' pathway of binding to nucleolar shuttling proteins, and those karyophilic proteins that function in other areas of the nucleus must provide additional signals to prevent their interaction with shuttling proteins, and subsequent nucleolar targeting. This mechanism would potentially enable the efficient import of ribosomal proteins directly to their site of assembly into ribosomal subunits.

4.4.4 Implications for coordinated nuclear import

5S rRNA, bound with ribosomal protein L5 as a 5S RNP complex, behaves like a protein in its pathway of import. This is in contrast to the nuclear import pathways of the two other small nuclear RNAs used in this study. U1 snRNA and U3 snoRNA both have an essential requirement for interaction between elements of RNA structure and the nuclear transport machinery, and do not utilise saturable components in common with karyophilic proteins (Michaud & Goldfarb, 1992), yet they are also complexed with proteins as RNPs during their import (Lührmann *et al.*, 1990, Baserga *et al.*, 1992). So, why do 5S RNPs have protein-like targeting signals? The answer to this question may lie in the role of 5S rRNA as an integral component of ribosomes. The synthesis of ribosomes requires the coordinated assembly of proteins and ribosomal RNAs at the nucleolus (Hadjiolov, 1985). 5S rRNA is the only ribosomal RNA that is stored in the cytoplasm in oocytes, and a separate mechanism of nuclear import than the ribosomal proteins with which it must combine would appear to be a waste of cellular resources. Several ribosomal proteins, such as yeast YL3 (Moreland *et al.*, 1985) and L25 (Schaap *et al.*, 1991), have been found to possess NLSs, suggesting that their nuclear import proceeds via the SV40 T antigen-saturable pathway. Employing the same pathway of import for all ribosomal components would also assist in the regulation of their concurrent nuclear (and nucleolar) localisation.

The processes of nuclear export of U1 snRNA and 5S rRNA appear to involve common transport factors, as the presence of the HIV-1 Rev protein inhibits the export of both RNAs (Fischer *et al.*, 1995). However, the results presented here demonstrate that there are different saturable factors involved in the nuclear import of the two molecules. It is possible that a more general mechanism is utilised for the export of U snRNAs and 5S rRNA; whereas, import is regulated by more specialised interactions, to allow for differential timing of import and regulation with other components of spliceosomes and ribosomes, respectively. This may include the utilisation different members of the NLS receptor gene family for specific NLS-receptor interactions (Adam, 1995; Weis *et al.*, 1995).

Given the nucleolar nature of U3 snoRNA, it was thought that it might utilise a similar pathway of nuclear import to 5S rRNA, another nucleolar-targeted molecule. However, it appears that U3 snoRNA is normally retained in the nucleus, and is matured in that cellular compartment without a cytoplasmic journey (Terns *et al.*, 1995). Therefore, the receptor-mediated import of U3 snoRNA into the nucleus may occur by a more obscure pathway, that does not require coordination with that of other nucleolar molecules.

4.4.5 Summary

The results presented here suggest that 5S rRNA is imported into the nucleus after cytoplasmic storage bound with ribosomal protein L5. It is possible that 5S rRNA has a passive role in its own transport and L5 contributes the targeting signal to the complex. In this scenario, 5S rRNA is imported by the same pathway as ribosomal proteins, which would aid in the coordination of the process of ribosome assembly.

Chapter Five
General Discussion

The results of the study outlined in this thesis emphasise the importance of specific RNA-protein interactions in directing the nucleo-cytoplasmic localisation of oocyte-type 5S rRNA during oogenesis in *Xenopus laevis*. Furthermore, the results of this investigation provide a model of 5S rRNA import which integrates its mobilisation to the nucleus with the pathway likely to be used by ribosomal proteins.

5.1 The Pathway for 5S rRNA Nuclear Import

Oocyte-type 5S rRNA moves between nuclear and cytoplasmic compartments throughout the stages of oogenesis, undergoing a period of storage in the cytoplasm prior to its assembly into ribosomal subunits. 5S rRNA is synthesised in the nucleus throughout oogenesis, but is transported to the cytoplasm to be stored as 42S RNPs and 7S RNPs in early stage oocytes (Honda & Roeder, 1980; Pelham & Brown, 1980; Picard *et al.*, 1980; Mattaj *et al.*, 1987; Joho *et al.*, 1990). The synthesis of TFIIIA, the protein component of 7S RNPs, is greatly reduced in the later stages of oocyte development during vitellogenesis, and the accumulation of the protein components of the 42S RNPs ceases entirely (Dixon & Ford, 1982). In vitellogenic oocytes, 18S-5.8S-28S ribosomal RNAs and proteins are synthesised and congregate in the nucleolus to take part in ribosome assembly (Mairy & Denis, 1971; Picard & Wegnez, 1979; Picard *et al.*, 1980; Dixon & Ford, 1982). Newly synthesised 5S rRNA accumulates in the nucleolus directly; however, stored 5S rRNA must be released from its retention in the cytoplasm to take part in the assembly of ribosomal subunits.

The signal for nuclear accumulation appears to come from displacement of bound TFIIIA from 5S rRNA, and its replacement with ribosomal protein L5. The resulting 5S RNP is a precursor to ribosome assembly (Steitz *et al.*, 1988; Allison *et al.*, 1991), and remains as a stable complex which can be released intact from the fully assembled ribosome by EDTA treatment (Blobel, 1971). The results of studies presented in this thesis suggest that ribosomal protein L5 provides the signal for

nuclear targeting of 5S RNPs. The yeast homologue of L5, YL3, possesses a sequence at its amino terminus which is sufficient to localise the non-nuclear protein β-galactosidase to the nucleus (Moreland *et al.*, 1985). Furthermore, sequence analysis of L5 reveals that it possesses a putative nuclear localisation signal between amino acid residues 255 and 264 (Wormington, 1989). However, direct functional analysis of this sequence as an NLS has not been performed.

The presence of the NLS on L5 would allow the 5S RNP complex to interact with a karyophilic protein-type NLS receptor, and to enter the nuclear import pathway. This receptor could be the importin-α part of the nuclear pore targeting complex (Görlich *et al.*, 1994; 1995a,b; Imamoto *et al.*, 1995a,b,c). After interaction with the 5S RNP, the nuclear pore targeting complex would dock at the nuclear pore complex, and be translocated through the NPC channel. These docking and/or translocation processes must involve interaction of the 5S RNP-nuclear pore targeting complex with *N*-acetylglucosamine-bearing nucleoporins, as binding of wheat germ agglutinin to these sites inhibits 5S RNP nuclear accumulation. 5S RNPs are likely to remain associated with the targeting complex until their arrival at the nucleoplasmic side of the NPC, where the importin-β component dissociates (Görlich *et al.*, 1995b). Prior to localisation to the nucleolus, or other potential retention sites in the nucleus, 5S RNPs would be released from the rest of the complex, which could then be recycled for use in nuclear export or import of other molecules.

The process of 5S RNP nuclear import is dependent on physiological temperatures and ATP. This temperature dependence probably reflects the many enzymes involved in the interactions between components along the nuclear transport pathway and in the mechanism promoting the dilation of the NPC channel to allow access to the 5S RNP. Similarly, the ATP requirements could be at any stage in the process leading to nuclear accumulation; from initial interaction with the NLS receptor, to interaction with a 5S RNP nuclear retention site such as at the nucleolus. The activation of the nuclear localisation sequence for 5S RNPs could require phosphorylation of the molecule by an ATP-dependent protein kinase (Rihs & Peters, 1989; Rihs *et al.*, 1991; Meßmer & Dreyer, 1993; Jans & Jans, 1994; Gauthier-Rouvière *et al.*, 1995; Vancurova *et al.*, 1995). In addition, the 5S RNP may need to undergo an ATP-dependent conformational change to enter the NPC, as has been

visualised for *Chironomus* RNPs (Mehlin *et al.*, 1992; 1995). Movement through the NPC channel also involves the use of metabolic energy. Recent studies have suggested that it is not necessarily ATP hydrolysis that is involved in the translocation mechanism, but GTP hydrolysis via the GTPase Ran and its associated proteins (Moore & Blobel, 1993; 1994a). However, is still possible that ATP is required for the regulation and activation of enzymes associated with the translocation process. Finally, the intranuclear movement of newly imported 5S RNPs from the NPC to the nucleolus may involve ATP. Although GTP is the nucleotide that has been implicated in the nucleolar localisation of U3 snoRNA (Cheng *et al.*, 1995), there is evidence of an ATP requirement for movement of the nucleolar protein No38 within the nucleus (Wu *et al.*, 1995b).

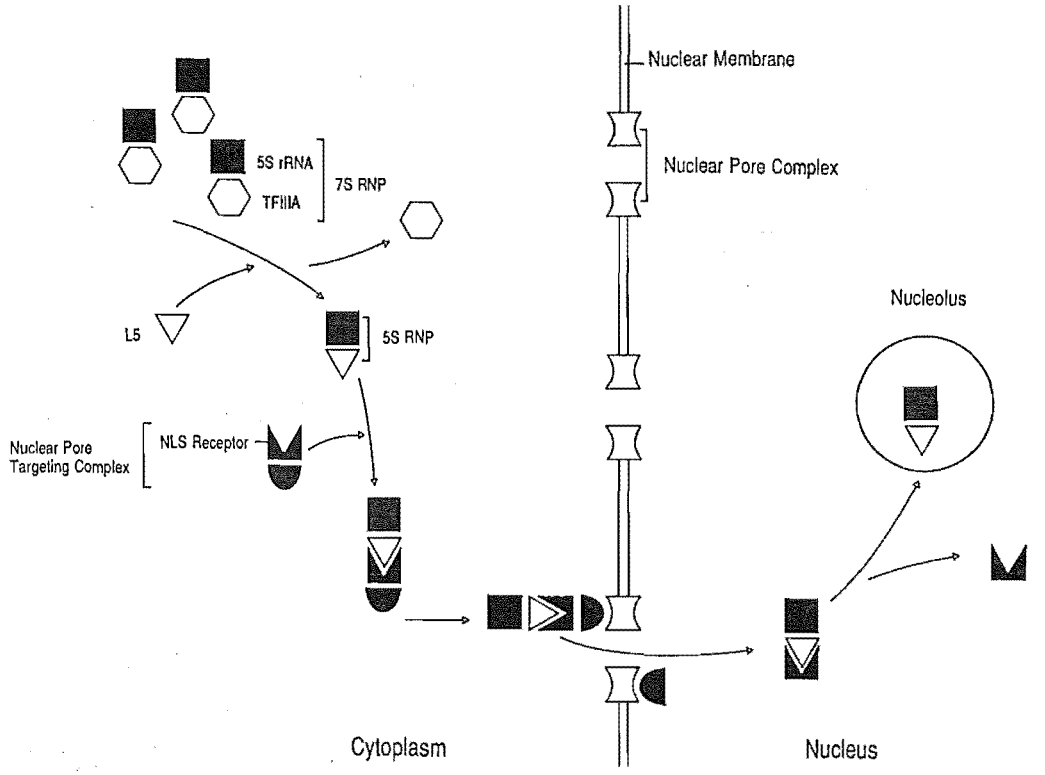
5.2 Shuttling

Some nucleolar proteins possess NLS-binding domains and are able to move between the nucleus and cytoplasm, such as Nopp140 (Meier & Blobel, 1990; 1992), No38 and nucleolin (Borer *et al.*, 1989). This has lead to the suggestion that these proteins could be involved in the nucleo-cytoplasmic trafficking of other nucleolar targeted molecules such as ribosomal proteins, although to date there has been no direct evidence of such a function (Borer *et al.*, 1989; Meier & Blobel, 1992; Xue & Mélèse, 1994). These so-called shuttling proteins would potentially collect protein or RNP cargo in the cytoplasm, move it through the import pathway to the nucleus, and subsequently deliver the transported molecule to the nucleolus, by the binding of the shuttling protein at this subnuclear compartment. Nuclear transport mediated by shuttling proteins provides an elegant explanation for nucleolar targeting of proteins and RNAs/RNPs, eliminating a requisite for individual signals to establish localisation to the nucleolus. This could result in an efficient and more easily regulated mode of transport to this compartment, compared with a scenario where each molecule has to be transported individually.

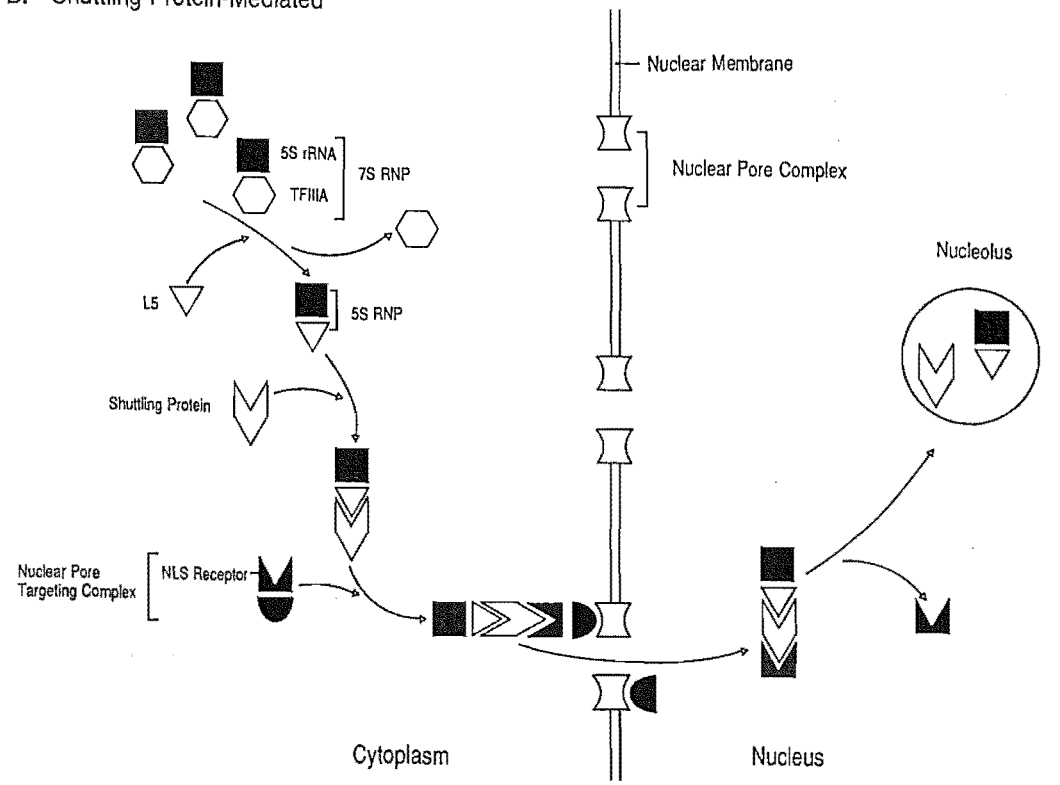
The inclusion of a nucleolar-based shuttling protein in the 5S RNP nuclear import pathway could explain both its nuclear and subsequent nucleolar localisation. Figure 5.1 illustrates two models for the pathway of 5S rRNA nuclear import; a direct

Figure 5.1: Models for the pathway of nuclear import of 5S rRNA in *Xenopus* oocytes.
(A) Direct nuclear import of 5S rRNA/5S RNPs. 5S rRNA is released from storage bound with the protein TFIIIA as 7S RNPs by the replacement of TFIIIA with ribosomal protein L5, forming 5S RNPs. The 5S RNP NLS, supplied by L5, binds to the NLS receptor component, probably importin-α (Görlich *et al.*, 1995a), of the nuclear pore targeting complex. The entire complex then undertakes the docking and translocation steps of transport through the NPC, a process involving sequential interaction with different regions of the NPC including *N*-acetylglucosamine-bearing nucleoporins. Once at the nucleoplasmic face of the NPC, the nuclear pore targeting complex dissociates, leaving at least the importin-β component in close proximity to the NPC (Görlich *et al.*, 1995b). The NLS-receptor is likely to be released from the transported 5S RNP prior to nucleolar localisation of the pre-ribosomal particle. The NLS receptor is then able to be recycled for use in nuclear export and/or import of new cargo molecules or complexes.
(B) Shuttling protein-mediated nuclear import of 5S rRNA/5S RNPs. This model involves the same interactions as described in (A), with the addition of a shuttling protein adaptor between the 5S RNP and the NLS-receptor of the nuclear pore targeting complex. The shuttling protein, which could be one of the three nucleolar proteins found to recycle between nucleus and cytoplasm (see text), binds to the NLS on L5. The shuttling protein-5S RNP complex could then interact with the nuclear pore targeting complex, via the NLS contained within the shuttling protein, and pass through the NPC. Once in the nucleus, the NLS receptor dissociates, and the shuttling protein aids in localisation of the shuttling protein-5S RNP complex to the nucleolus by its interaction with nucleolar components. 5S RNPs would next be released and bind to a specific site at the nucleolus to take part in ribosome assembly. The shuttling protein could potentially be recycled for use in nuclear export, possibly for ribosomal subunits.

A. Direct



B. Shuttling Protein-Mediated



(A) and a shuttling (B) scenario. The first step in the shuttling model of 5S RNP nuclear import involves cytoplasmic 5S RNPs binding the NLS-binding portion of the shuttling protein. The *Xenopus* homologue of rat Nopp140 has recently been isolated and cloned (Cairns & McStay, 1995), and nucleolin (Meßmer & Dreyer, 1993) and No38 (Schmidt-Zachmann *et al.*, 1987) have previously been isolated from *Xenopus*, providing three options for potential shuttling proteins in oocytes. In the next step of the shuttling model, after binding in the cytoplasm, the 5S RNP-shuttling protein complex subsequently interacts with the nuclear pore targeting complex by the binding of the shuttling protein NLS with the importin-α type NLS receptor. The entire complex then undergoes the docking and translocation steps of nuclear import. Once in the nucleus, the 5S RNP-shuttling protein complex moves through the nucleoplasm to bind to components of the nucleolus. The initial nucleolar interaction is likely to be achieved via the interaction of the shuttling protein with molecules in the nucleolus, such as rRNA. 5S RNPs would then be in proximity to nucleolar components to which they can bind to contribute to the assembly of 60S ribosomal subunits.

Phosphorylation of the putative shuttling protein B23/No38 has been shown to enhance its ability to bind NLSs (Szebeni *et al.*, 1995). Therefore, the ATP requirement of the 5S RNP nuclear transport process could be due in part to the ATP-dependent phosphorylation of the shuttling protein. In the absence of ATP, the 5S RNP NLS may be unable to interact with the NLS receptor on the shuttling protein, and would therefore be unable to be transported.

5.3 Coordination of Ribosome Assembly

Oocyte-type 5S rRNA is separated from the other ribosomal RNAs in *Xenopus* oocytes both temporally and spatially, in that it is synthesised from an earlier stage in oogenesis, and it resides in the cytoplasm until required. It would appear to be an inefficient use of cellular resources to establish an independent pathway for the nuclear import of 5S rRNA, especially as this RNA is able to form a complex with a ribosomal protein that is imported concurrently, and with which 5S rRNA remains bound within the functioning ribosome. Furthermore, a general mechanism is likely to

exist for the nuclear import of ribosomal proteins, as most nuclear proteins are imported by a pathway that can be saturated by a prototypical NLS (Michaud & Goldfarb, 1993). This 'general' mechanism would aid in the efficient simultaneous import of the many different proteins that are essential for ribosome assembly. It appears that 5S rRNA is 'piggy-backed' into the nucleus via this pathway by L5. This enables it to be mobilised at vitellogenesis during the maximal synthesis of other ribosomal components, which in the cytoplasm are the ribosomal proteins. It is possible that other processes requiring coordination of nuclear transport, such as elements of the splicing machinery for pre-mRNA and transcription factors for a group of genes that need to be expressed simultaneously, utilise common transport pathways in a similar manner.

5.4 Prospects and Conclusions

Further definition of the 5S rRNA/5S RNP nuclear import pathway is required to unequivocally establish the role of ribosomal protein L5 in this process. Mutational analyses of L5 would be invaluable in the verification of a direct role for this protein in 5S rRNA nuclear import. Through microinjection assays of *in vitro* produced L5 mutants lacking the putative NLS and which are still able to bind oocyte-type 5S rRNA, the targeting signal for the 5S RNP could be established. It would also be possible to test the ability of the 5S RNP to bind isolated NLS-receptors, including importin and putative shuttling proteins, to establish the molecular interactions involved in the transport pathway. Furthermore, it would be interesting to establish whether retention by non-diffusible elements plays a role in the nuclear accumulation of 5S RNPs. There is evidence that 5S RNPs are retained in the nucleus at sites apart from the nucleolus (M. North, pers. comm.); however, the nature of the retention complexes is unknown. This non-nucleolar pool of 5S RNPs in the nucleus may be a reserve ensuring that there is a constant supply of this essential ribosomal component during the rapid synthesis of ribosomes that accompanies oocyte development.

The nucleo-cytoplasmic transport of 5S rRNA in *Xenopus* oocytes throughout oogenesis is a highly regulated process resulting from differential association of the RNA with specific proteins. This mechanism underscores the importance of RNA-

protein interactions in creating the diversity of signals that coordinate nucleo-cytoplasmic transport with other cellular processes throughout development. Furthermore, the discovery that 5S rRNA utilises the same pathway of nuclear import as karyophilic proteins reinforces the connection between the transport processes of RNAs and proteins. Through interaction with nuclear transport factors and adaptor molecules such as shuttling proteins, the nucleo-cytoplasmic transport of RNA becomes a phenomenon of protein transport. Additionally, after initial interaction between NLSs and NLS receptors, many different types of RNAs and proteins may bind the same nuclear transport factors. It is possible that the many different molecules and complexes that move between the nucleus and cytoplasm converge on a limited number of nuclear transport pathways, due to interaction with these common transport intermediates. Therefore, a definitive model for nucleo-cytoplasmic transport of proteins and RNAs may turn out to be much simpler than might be imagined from the differences between these multifarious molecules.

Acknowledgements

I wish to express my thanks to my supervisor Dr Lizabeth Allison for her support of my work, and aiding in its presentation throughout the world at conferences. Thanks also to my associate supervisor Dr Drusilla Mason for reading the final draft and for helpful discussions throughout this thesis. I was able to undertake this thesis work due to a Study Award for Women in Science from the Ministry of Research, Science and Technology.

I presented part of the work outlined in this thesis at the Symposium on RNA Processing and Nucleo-Cytoplasmic Transport in Marburg, Germany, in October 1993. My trip was made possible through a grant from the Peter Karlson Foundation (Germany) and the generous support of the Lührmann group at the Institut für Molekularbiologie und Tumorforschung, Phillips Universität Marburg. I am especially grateful to Dr Berthold Kastner for his help in getting me this financial assistance and for his warm hospitality. Thankyou also to Prof Tomas Pieler and Prof Reimer Stick of the Institut für Biochemie und Molekulare Zellbiologie, Göttingen, for their hospitality and discussions about my work. I received additional funding for this trip from the Canterbury Branches of the Royal Society of New Zealand and the New Zealand Federation of University Women.

Thankyou to those colleagues whom I have never met, who gave their time to produce, and send across the world, DNA, protein and protocols: Kara Reynolds of the University of Rochester (New York, USA) for the synthesis of RNase-free BSA, and Michael Ezrokhi of Brown University (Providence, Rhode Island, USA) for the production of the U3 snoRNA template. I also want to mention the assistance of members of the Zoology staff, especially Terry Williams for his perseverance with the photographic figures, and Jan MacKenzie for the fluorescence microscopy.

I am indebted to my friends from the Zoology Department who listened to, and understood, the frustrations of biological experiments and the endless repetitions of late night assays: Jane Symonds, Barbara Schakau, Jenny Khoo, Seumas Walker, Debbie Hume and Andrew Catanach. A special acknowledgement goes to Melanie North, for her inspiring discussions about 5S rRNA and for the critical reading of the main paper produced from this thesis. I owe a great deal to my many other friends for their ongoing support and warmth, in particular Eunice Chinnery, Susan Hood, Joanne Drayton and Susan Marshall. Thanks also to my grandmother, Gladys Murdoch, for always being there for me, and to my parents for placing an importance on education.

Finally, much love and many, many thanks to Nicole Beger-Hintzen for her patient practical help with the production of this thesis (particularly the creation of Figure 5.1) and especially for her love and care which enabled me to enjoy life immensely at the same time as completing this work.

References

Adam, S.A. 1995. The importance of importin. *Trends Cell Biol.* **5**: 189-191.

Adam, E.J.H., & S.A. Adam. 1994. Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope. *J. Cell Biol.* **125**: 547-555.

Adam, S.A., & L. Gerace. 1991. Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. *Cell* **66**: 837-847.

Adam, S.A., T.J. Lobl, M.A. Mitchell, & L. Gerace. 1989. Identification of specific binding proteins for a nuclear location sequence. *Nature* **337**: 276-279.

Adam, S.A., R. Sterne-Marr, & L. Gerace. 1990. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* **111**: 807-816.

Agutter, P.S., & D. Prochnow. 1994. Nucleocytoplasmic transport. *Biochem. J.* **300**: 609-618.

Agutter, P.S., B. McCaldin, & H.J. McArdle. 1979. Importance of mammalian nuclear-envelope nucleoside triphosphatase in nucleo-cytoplasmic transport of ribonucleoproteins. *Biochem. J.* **182**: 811-819.

Akey, C.W. 1989. Interactions and structure of the nuclear pore complex revealed by cryo-electron microscopy. *J. Cell Biol.* **109**: 955-970.

Akey, C.W. 1990. Visualization of transport-related configurations of the nuclear pore transporter. *Biophys. J.* **58**: 341-355.

Akey, C.W. 1992. The nuclear pore complex: a macromolecular transporter. In: **Feldherr, C.M.** (ed). *Nuclear Trafficking*. San Diego. Academic Press: 31-70.

Akey, C.W., & D.S. Goldfarb. 1989. Protein import through the nuclear pore complex is a multistep process. *J. Cell Biol.* **109**: 971-982.

Allen, E.D. 1990. Pores of annulate lamellae and nuclei bind wheat germ agglutinin and monoclonal antibody similarly. *J. Struct. Biol.* **103**: 140-151.

Allison, L.A. 1989. Fate of stored 5S RNA during ribosome biogenesis in oocytes of *Xenopus laevis*. PhD Thesis. University of Washington, Seattle, Washington, USA.

Allison, L.A., P.J. Romaniuk, & A.H. Bakken. 1991. RNA-protein interactions of stored 5S RNA with TFIIIA and ribosomal protein L5 during *Xenopus* oogenesis. *Devel. Biol.* **144**: 129-144.

Allison, L.A., M.T. North, K.J. Murdoch, P.J. Romaniuk, S. Deschamps, & M. le Maire. 1993. Structural requirements of 5S rRNA for nuclear transport, 7S ribonucleoprotein particle assembly, and 60S ribosomal subunit assembly in *Xenopus* oocytes. *Mol. Cell. Biol.* **13**: 6819-6831.

Allison, L.A., M.T. North, & L.A. Neville. 1995. Differential binding of oocyte-type and somatic-type 5S rRNA to TFIIIA and ribosomal protein L5 in *Xenopus* oocytes: specialization for storage versus mobilization. *Devel. Biol.* **168**: 284-295.

Amberg, D.C., M. Fleishmann, I. Stagljar, C.N. Cole, & M. Aebi. 1993. Nuclear PRP20 protein is required for mRNA export. *EMBO J.* **12**: 233-241.

Baglia, F.A., & G.G. Maul. 1983. Nuclear ribonucleoprotein release and nucleoside triphosphatase activity are inhibited by antibodies directed against one nuclear matrix glycoprotein. *Proc. Natl. Acad. Sci. USA* **80**: 2285-2289.

Baserga, S.J., M. Gilmore-Hebert, & X. W. Yang. 1992. Distinct molecular signals for nuclear import of the nucleolar snRNA, U3. *Genes Devel.* **6**: 1120-1130.

Bataillé, N., T. Helser, & H.M. Fried. 1990. Cytoplasmic transport of ribosomal subunits microinjected into the *Xenopus laevis* oocyte nucleus: a generalized, facilitated process. *J. Cell Biol.* **111**: 1571-1582.

Benditt, J.O., C. Meyer, H. Fasold, F.C. Barnard, & N. Riedel. 1989. Interaction of a nuclear location signal with isolated nuclear envelopes and identification of signal-binding proteins by photoaffinity labeling. *Proc. Natl. Acad. Sci. USA* **86**: 9327-9331.

Bernd, A., H.C. Schröder, R.K. Zahn, & W.E.G. Müller. 1982. Modulation of the nuclear-envelope nucleoside triphosphatase by Poly(A)-rich mRNA and by microtubule protein. *Eur. J. Biochem.* **129**: 43-49.

Berrios, M. 1992. Nuclear pore complex-associated ATPase. In: **Feldherr, C.M.** (ed). *Nuclear Trafficking*. San Diego, Academic Press: 203-227.

Berrios, M., & P.A. Fisher. 1986. A myosin heavy chain-like polypeptide is associated with the nuclear envelope in higher eukaryotic cells. *J. Cell Biol.* **103**: 711-724.

Bischoff, F.R., H. Krebber, T. Kempf, I. Hermes, & H. Ponstingl. 1995a. Human RanGTPase-activating protein RanGAP1 is a homologue of yeast Rna1p involved in mRNA processing and transport. *Proc. Natl. Acad. Sci. USA* **92**: 1749-1753.

Bischoff, F.R., H. Krebber, E. Smirnova, W. Dong, & H. Ponstingl. 1995b. Co-activation of RanGTPase and inhibition of GTP dissociation by RAN-GTP binding protein RanBP1. *EMBO J.* **14**: 705-715.

Blobel, G. 1971. Isolation of a 5S RNA-protein complex from mammalian ribosomes. *Proc. Natl. Acad. Sci. USA* **68**: 1881-1885.

Boelens, W.C., I. Palacios, & I.W. Mattaj. 1995. Nuclear retention of RNA as a mechanism for localization. *RNA* **1**: 273-283.

Bogerd, H.P., R.A. Fridell, S. Madore, & B.R. Cullen. 1995. Identification of a novel cellular cofactor for the Rev/Rex class of retroviral regulatory proteins. *Cell* **82**: 485-494.

Borer, R.A., C.F. Lehner, H.M. Eppenberger, & E.A. Nigg. 1989. Major nucleolar proteins shuttle between nucleus and cytoplasm. *Cell* **56**: 379-390.

Breeuwer, M, & D.S. Goldfarb. 1990. Facilitated transport of histone H1 and other small nucleophilic proteins. *Cell* **60**: 999-1008.

Bustamante, J.O. 1992. Nuclear ion channels in cardiac myocytes. *Pfl. Arch.* **421**: 473-485.

Bustamante, J.O. 1994. Nuclear electrophysiology. *J. Membr. Biol.* **138**: 105-112.

Cairns, C., & B. McStay. 1995. Identification and cDNA cloning of a *Xenopus* nucleolar phosphoprotein, xNopp180, that is the homolog of the rat nucleolar protein Nopp140. *J. Cell Sci.* **108**: 3339-3347.

Callaci, T.P., G-Z. Cai, J.C. Lee, T.J. Daly, & C-W. Wu. 1990. Assembly of *Xenopus* transcription factor III A-5S RNA complex. *Biochem.* **29**: 4653-4659.

Cheng, Y., J.E. Dahlberg, & E. Lund. 1995. Diverse effects of the guanine nucleotide exchange factor RCC1 on RNA transport. *Science* **267**: 1807-1810.

Chi, N.C., E.J.H. Adam, & S.A. Adam. 1995. Sequence and characterization of cytoplasmic nuclear protein import factor p97. *J. Cell Biol.* **130**: 265-274.

Cohen, R.J., & P.L. Paine. 1992. Biophysics of nucleocytoplasmic transport. In: **Feldherr, C.M.** (ed). *Nuclear Trafficking*. San Diego, Academic Press: 333-362.

Corbett, A.H., D.M. Koepp, G. Schlenstedt, M.S. Lee, A.K. Hopper. & P.A. Silver. 1995. Rna1p, a Ran/TC4 GTPase activating protein, is required for nuclear import. *J. Cell Biol.* **130**: 1017-1026.

Cordes, V.I., I. Waizenegger, & G. Krohne. 1991. Nuclear pore complex glycoprotein p62 of *Xenopus laevis* and mouse: cDNA cloning and identification of its glycosylated region. *Eur. J. Cell Biol.* **55**: 31-47.

Cordes, V.C., S. Reidenbach, A. Köhler, N. Stuurman, R. van Driel, & W.W. Franke. 1993. Intranuclear filaments containing a nuclear pore complex protein. *J. Cell Biol.* **123**: 1333-1344.

Dabauvalle, M-C., B. Schulz, U. Scheer, & R. Peters. 1988. Inhibition of nuclear accumulation of karyophilic proteins in living cells by microinjection of the lectin wheat germ agglutinin. *Exp. Cell Res.* **174**: 291-296.

Dang, C.V., & W.M.F. Lee. 1989. Nuclear and nucleolar targeting sequences of c-*erb*-A, c-*myb*, N-*myc*, p53, HSP70, and HIV *tat* proteins. *J. Biol. Chem.* **264**: 18019-18023.

Darby, M.K, & K.E. Joho. 1992. Differential binding of zinc fingers from *Xenopus* TFIIIA and p43 to 5S RNA and the 5S RNA gene. *Mol. Cell. Biol.* **12**: 3155-3164.

Dargemont, C., & L.C. Kühn. 1992. Export of mRNA from microinjected nuclei of *Xenopus laevis* oocytes. *J. Cell Biol.* **118**: 1-9.

Dargemont, C., M.S. Schmidt-Zachmann, & L.C. Kühn. 1995. Direct interaction of nucleoporin p62 with mRNA during its export from the nucleus. *J. Cell Sci.* **108**: 257-263.

Dasso, M. 1993. RCC1 in the cell cycle: the regulator of chromosome condensation takes on new roles. *Trends Biochem. Sci.* **18**: 96-101.

Davis, L.I. 1995. The nuclear pore complex. *Annu. Rev. Biochem.* **64**: 865-896.

Davis, L.I., & G. Blobel. 1986. Identification and characterization of a nuclear pore complex protein. *Cell* **45**: 699-709.

Davis, L.I., & G. Blobel. 1987. Nuclear pore complex contains a family of glycoproteins that includes p62: glycosylation through a previously unidentified cellular pathway. *Proc. Natl. Acad. Sci. USA* **84**: 7552-7556.

Dean, D.A., & H. Kasamatsu. 1994. Signal- and energy-dependent nuclear transport of SV40 Vp3 by isolated nuclei. *J. Biol. Chem.* **269**: 4910-4916.

Denis, H., & M. Wegnez. 1977. Biochemical research on oogenesis. Oocytes of *Xenopus laevis* synthesise but do not accumulate 5S RNA of somatic type. *Devel. Biol.* **58**: 212-217.

De Robertis, E.M., S. Lienhard, R.F. Parisot. 1982. Intracellular transport of microinjected 5S and small nuclear RNAs. *Nature* **295**: 572-577.

Dingwall, C., S.V. Sharnick, & R.A. Laskey. 1988. A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. *Cell* **30**: 449-458.

Dingwall, C., S. Kandels-Lewis, & B. Séraphin. 1995. A familiy of Ran binding proteins that includes nucleoporins. *Proc. Natl. Acad. Sci. USA* **92**: 7525-7529.

Dixon, L.K., & P.J. Ford. 1982. Regulation of protein synthesis and accumulation during oogenesis in *Xenopus laevis*. *Devel. Biol.* **93**: 478-497.

Doye, V., & E.C. Hurt. 1995. Genetic approaches to nuclear pore structure and function. *Trends Genetics* **11**: 235-241.

Dreyer, C. 1989. Fate and nuclear localization of germinal vesicle proteins during embryogenesis. *Devel. Biol.* **6**: 31-57.

Dreyfuss, G., L. Philipson, & I.W. Mattaj. 1988. Ribonucleoprotein particles in cellular processes. *J. Cell Biol.* **106**: 1419-1425.

Dumont, J.N. 1972. Oogenesis in *Xenopus laevis* (Daudin). 1. Stages of oocyte development in laboratory maintained animals. *J. Morph.* **136**: 153-180.

Dworetzky, S.I., & C.M. Feldherr. 1988. Translocation of RNA-coated gold particles through the nuclear pores of oocytes. *J. Cell Biol.* **106**: 575-584.

Dworetzky, S.I., R.E. Lanford, & C.M. Feldherr. 1988. The effects of variations in the number and sequence of targeting signals on nuclear uptake. *J. Cell Biol.* **107**: 1279-1287.

Eckner, R., W. Ellmeier, & M.L. Birnstiel. 1991. Mature 3′ end formation stimulates RNA export from the nucleus. *EMBO J.* **10**: 3513-3522.

Enenkel, C., G. Blobel, & M. Rexach. 1995. Identification of a yeast karyopherin heterodimer that targets import substrate to mammalian nuclear pore complexes. *J. Biol. Chem.* **270**: 16499-16502.

Fabre, E., & E.C. Hurt. 1994. Nuclear transport. *Curr. Opin. Cell Biol.* **6**: 335-342.

Featherstone, C., M.K. Darby, & L. Gerace. 1988. A monoclonal antibody against the nuclear pore complex inhibits nucleocytoplasmic transport of protein and RNA in vivo. *J. Cell Biol.* **107**: 1289-1297.

Feldherr, C.M., E. Kallenbach, & N. Schultz. 1984. Movement of a karyophilic protein through the nuclear pores of oocytes. *J. Cell Biol.* **99**: 2216-2222.

Finch, R.A., G.R. Renvankar, & P.K. Chan. 1993. Nucleolar localization of nucleophosmin/B23 requires GTP. *J. Biol. Chem.* **268**: 5823-5827.

Finlay, D.R., & D.J. Forbes. 1990. Reconstitution of biochemically altered nuclear pores: transport can be eliminated and restored. *Cell* **60**: 17-29.

Finlay, D.R., D.D. Newmeyer, T.M. Price, & D.J. Forbes. 1987. Inhibition of in vitro nuclear transport by a lectin that binds to nuclear pores. *J. Cell Biol.* **104**: 189-200.

Finlay, D.R., E. Meier, P. Bradley, J. Horecka, & D.J. Forbes. 1991. A complex of nuclear pore proteins required for pore function. *J. Cell Biol.* **114**: 169-183.

Fischer, U., & R. Lührmann. 1990. An essential signalling role for the m_3G cap in the transport of U1 snRNA to the nucleus. *Science* **249**: 786-790.

Fischer, U., E. Darzynkiewicz, S.M. Tahara, N.A. Dathan, R. Lührmann, & I.W. Mattaj. 1991. Diversity in the signals required for nuclear accumulation of U snRNPs and variety in the pathways of nuclear transport. *J. Cell Biol.* **113**: 705-714.

Fischer, U., V. Sumpter, M. Sekine, T. Satoh, & R. Lührmann. 1993. Nucleo-cytoplasmic transport of U snRNPs: definition of a nuclear location signal in the Sm core domain that binds a transport receptor independently of the m_3G cap. *EMBO J.* **12**: 573-583.

Fischer, U., S. Meyer, M. Teufel, C. Heckel, R. Lührmann, & G. Rautmann. 1994a. Evidence that HIV-1 Rev directly promotes the nuclear export of unspliced RNA. *EMBO J.* **13**: 4105-4112.

Fischer, U., J. Heinrich, K. van Zee, E. Fanning, & R. Lührmann. 1994b. Nuclear transport of U1 snRNP in somatic cells: differences in signal requirement compared with *Xenopus laevis* oocytes. *J. Cell Biol.* **125**: 971-980.

Fischer, U., J. Huber, W.C Boelens, I.W. Mattaj, & R. Lührmann. 1995. The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell* **82**: 475-483.

Flach, J., M. Bossie, J. Vogel, A. Corbett, T. Jinks, D.A. Willins, & P.A. Silver. 1994. A yeast RNA-binding protein shuttles between the nucleus and the cytoplasm. *Mol. Cell. Biol.* **14**: 8399-8407.

Forbes, D.J. 1992. Structure and function of the nuclear pore complex. *Annu. Rev. Cell Biol.* **8**: 495-527.

Ford, P.J., & E.M. Southern. 1973. Differential sequences for 5S RNA in kidney cells and ovaries of *Xenopus laevis*. *Nature New Biol.* **241**: 7-12.

Garcia-Bustos, J., J. Heitman, & M.N. Hall. 1991a. Nuclear protein localization. *Biochim. Biophys. Acta* **1071**: 83-101.

Garcia-Bustos, J.F., P. Wagner, & M.N. Hall. 1991b. Yeast cell-free nuclear protein import requires ATP hydrolysis. *Exp. Cell Res.* **192**: 213-219.

Gauthier-Rouvière, C., M. Vandromme, N. Lautredou, Q.Q. Cai, F. Girard, A. Fernandez, & N. Lamb. 1995. The serum response factor nuclear localization signal: general implications for cyclic AMP-dependent protein kinase activity in control of nuclear translocation. *Mol. Cell. Biol.* **15**: 433-444.

Gerace, L. 1995. Nuclear export signals and the fast track to the cytoplasm. *Cell* **82**: 341-344.

Ginsberg, A.M., B.O. King, & R.G. Roeder. 1984. *Xenopus* 5S gene transcription factor, TFIIIA: characterization of a cDNA clone and measurement of RNA levels throughout development. *Cell* **39**: 479-489.

Glibetic, M., D.E. Larson, N. Sienna, J-P. Bachellerie, & B.H. Sells. 1992. Regulation of U3 snRNA expression during myoblast differentiation. *Exp. Cell Res.* **202**: 183-189.

Goldberg, M.W., & T.D. Allen. 1992. High resolution scanning electron microscopy of the nuclear envelope: demonstration of a new, regular, fibrous lattice attached to the baskets of the nucleoplasmic face of the nuclear pores. *J. Cell Biol.* **119**: 1429-1440.

Goldberg, M.W., & T.D. Allen. 1993. The nuclear pore complex: three-dimensional surface structure revealed by field emission, in-lens scanning electron microscopy, with underlying structure uncovered by proteolysis. *J. Cell Sci.* **106**: 261-274.

Goldfarb, D.S. 1994. GTPase cycle for nuclear transport. *Curr. Biol.* **4**: 57-60.

Goldfarb, D.S., J. Gariepy, G. Schoolnik, & R.D. Kornberg. 1986. Synthetic peptides as nuclear localization signals. *Nature* **322**: 641-644.

Görlich, D., S. Prehn, R.A. Laskey, & E. Hartmann. 1994. Isolation of a protein that is essential for the first step of protein import. *Cell* **79**: 767-778.

Görlich, D., S. Kostka, R. Kraft, C. Dingwall, R.A. Laskey, E. Hartmann, & S. Prehn. 1995a. Two different subunits of importin cooperate to recognise nuclear localisation signals and bind them to the nuclear envelope. *Curr. Biol.* **5**: 383-392.

Görlich, D., F. Vogel, A.D. Mills, E. Hartmann, & R.A. Laskey. 1995b. Distinct functions for the two importin subunits in nuclear protein import. *Nature* **377**: 246-248.

Guddat, U., A.H. Bakken, & T. Pieler. 1990. Protein-mediated nuclear export of RNA: 5S rRNA containing small RNPs in *Xenopus* oocytes. *Cell* **60**: 619-628.

Guiochon-Mantel, A., P. Lescop, S. Christin-Maitre, H. Loosfelt, M. Perrot-Applanat, & E. Milgrom. 1991. Nucleocytoplasmic shuttling of the progesterone receptor. *EMBO J.* **10**: 3851-3859.

Guiochon-Mantel, A., K. DeLabre, P. Lescop, & E. Milgrom. 1994. Nuclear localization signals also mediate the outward movement of proteins from the nucleus. *Proc. Natl. Acad. Sci. USA* **91**: 7179-7183.

Gulizia, J., M.P. Dempsey, N. Sharova, M.I. Bukrinsky, L. Spitz, D. Goldfarb, & M. Stevenson. 1994. Reduced nuclear transport of human immunodeficiency virus type 1 preintegration complexes in the presence of a prototypic nuclear targeting signal. *J. Virol.* **68**: 2021-2025.

Hadjiolov, A.A. 1985. *The Nucleolus and Ribosome Biogenesis*. Springer-Verlag, New York.

Hamm, J., & I.W. Mattaj. 1990. Monomethylated cap structures facilitate RNA export from the nucleus. *Cell* **63**: 109-118.

Hamm, J., M. Kazmaier, & I.W. Mattaj. 1987. *In vitro* assembly of U1 snRNPs. *EMBO J.* **6**: 3479-3485.

Hamm, J., E. Darzynkiewicz, S.M. Tahara, & I.W. Mattaj. 1990. The trimethylguanosine cap structure of U1 snRNA is a component of a bipartite nuclear targeting signal. *Cell* **62**: 569-577.

Hanover, J.A., C.K. Cohen, M.C. Willingham, & M.K. Park. 1987. O-linked N-acetylglucosamine is attached to proteins of the nuclear pore. *J. Biol. Chem.* **262**: 9887-9894.

Harootunian, A.T., S.R. Adams, W. Wen, J.L. Meinkoth, S.S. Taylar, & R.Y. Tsien. 1993. Movement of the free catalytic subunit of cAMP-dependent protein kinase into and out of the nucleus can be explained by diffusion. *Mol. Biol. Cell* **4**: 993-1002.

Hart, G.W., R.S. Haltiwanger, G.D. Holt, & W.G. Kelly. 1989. Glycosylation in the nucleus and cytoplasm. *Annu. Rev. Biochem.* **58**: 841-874.

Hartmann, E., & D. Görlich. 1995. A Ran-binding motif in nuclear pore proteins. *Trends Cell Biol.* **5**: 192-193.

Hausen, P., & M. Riebsell. 1991. *The Early Development of Xenopus laevis: An Atlas of the Histology.* Springer Verlag, Berlin.

He, X-P., N. Battaillé, & H.M. Fried. 1994. Nuclear export of signal recognition particle RNA is a facilitated process that involves the *Alu* sequence domain. *J. Cell Sci.* **107**: 903-912.

Hicks, G.R., & N.V. Raikhel. 1995. Nuclear localization signal binding proteins in higher plant nuclei. *Proc. Natl. Acad. Sci. USA* **92**: 734-738.

Hicks, G.R., H.M.S. Smith, M. Shieh, & N.V. Raikhel. 1995. Three classes of nuclear import signals bind to plant nuclei. *Plant Physiol.* **107**: 1055-1058.

Hill, W.E., A. Dahlberg, R.A. Garrett, P.B. Moore, D. Schlessinger, & J.R. Warner (eds). 1990. *The Ribosome: Structure, Function and Evolution.* Washington, DC, American Society for Microbiology.

Himpens, B., H. De Smedt, & R. Casteels. 1994a. Relationship between [Ca^{2+}] changes in the nucleus and cytosol. *Cell Calcium* **16**: 239-246.

Himpens, B., H. De Smedt, & M. Bollen. 1994b. Modulation of nucleocytosolic [Ca^{2+}] gradient in smooth muscle by protein phosphorylation. *FASEB J.* **8**: 879-883.

Hinshaw, J.E. 1994. Architecture of the nuclear pore complex and its involvement in nucleocytoplasmic transport. *Biochem. Pharmacol.* **47**: 15-20.

Hinshaw, J.E., B.O. Carragher, & R.A. Milligan. 1992. Architecture and design of the nuclear pore complex. *Cell* **69**: 1133-1141.

Holt, G.D., & G.W. Hart. 1986. The subcellular distribution of terminal *N*-acetylglucosamine moieties. Localization of a novel protein-saccharide linkage, O-linked GlcNac. *J. Biol. Chem.* **261**: 8049-8057.

Holt, G.D., C.M. Snow, A. Senior, R.S. Haltiwanger, L. Gerace, & G.W. Hart. 1987. Nuclear pore complex glycoproteins contain cytoplasmically disposed O-linked-*N*-acetylglucosamine. *J. Cell Biol.* **104**: 1157-1164.

Honda, B.M., & R.G. Roeder. 1980. Association of a 5S gene transcription factor with 5S RNA and altered levels of the factor during cell differentiation. *Cell* **22**: 119-126.

Huang, S., T.J. Deerinck, M.H. Ellisman, & D.L. Spector. 1994. In vivo analysis of nuclear poly(A)$^+$RNA. *J. Cell Biol.* **126**: 877-899.

Huber., P.W., & I.G. Wool. 1986. Use of the cytotoxic nuclease α-sarcin to identify the binding site on eukaryotic 5S ribosomal ribonucleic acid for the ribosomal protein L5. *J. Biol. Chem.* **261**: 3002-3005.

Hurt, E.C. 1993. The nuclear pore complex. *FEBS Lett.* **325**: 76-80.

Imamoto, N., Y. Matsuoka, T. Kurihara, K. Kohno, M. Miyagi, F. Sakiyama, Y. Okada, S. Tsunasawa, & Y. Yoneda. 1992. Antibodies against 70-kD heat shock cognate protein inhibit mediated nuclear import of karyophilic proteins. *J. Cell Biol.* **119**: 1047-1061.

Imamoto, N., T. Shimamoto, S. Kose, T. Takao, T. Tachibana, M. Matsubae, T. Sekimoto, Y. Shimonishi, & Y. Yoneda. 1995a. Nuclear pore-targeting complex binds to nuclear pores after association with a karyophile. *FEBS Lett.* **368**: 415-419.

Imamoto, N., T. Shimamoto, T. Takao, T. Tachibana, S. Kose, M. Matsubae, T. Sekimoto, Y. Shimonishi, & Y. Yoneda. 1995b. *In vivo* evidence for involvement of a 58 kDa component of nuclear pore targeting complex in nuclear protein import. *EMBO J.* **14**: 3617-3626.

Imamoto, N., T. Tachibana, M. Matsubae, & Y. Yoneda. 1995c. A karyophilic protein forms a stable complex with cytoplasmic components prior to nuclear pore binding. *J. Biol. Chem.* **270**: 8559-8565.

Izaurralde, E., & I.W. Mattaj. 1992. Transport of RNA between nucleus and cytoplasm. *Semin. Cell Biol.* **3**: 279-288.

Izaurralde, E., & I.W. Mattaj. 1995. RNA export. *Cell* **81**: 153-159.

Izaurralde, E., J. Lewis, C. Gamberi, A. Jarmolowski, C. McGuigan, & I.W. Mattaj. 1995. A cap-binding protein complex mediating U snRNA export. *Nature* **376**: 709-712.

Jans, D.A. 1994. Nuclear signaling pathways for polypeptide ligands and their membrane receptors? *FASEB J.* **8**: 841-847.

Jans, D.A., & P. Jans. 1994. Negative charge at the casein kinase II site flanking the nuclear localization signal of the SV40 large T-antigen is mechanistically important for enhanced nuclear transport. *Oncogene* **9**: 2961-2968.

Jarmolowski, A., W.C. Boelens, E. Izaurralde, & I. Mattaj. 1994. Nuclear export of different classes of RNA is mediated by specific factors. *J. Cell Biol.* **124**: 627-635.

Jarnik, M., & U. Aebi. 1991. Toward a more complete 3-D structure of the nuclear pore complex. *J. Struct. Biol.* **107**: 291-308.

Joho, K.E., M.K. Darby, E.T. Crawford, & D.D. Brown. 1990. A finger protein structurally similar to TFIIIA that binds exclusively to 5S RNA in *Xenopus*. *Cell* **61**: 293-300.

Kadowaki, T., D. Goldfarb, L.M. Spitz, A.M. Tartakoff, & M. Ohno. 1993. Regulation of RNA processing and transport by a nuclear guanine nucleotide release protein and members of the Ras superfamily. *EMBO J.* **12**: 2929-2937.

Kalderon, D., B.L. Roberts, W.D. Richardson, & A.E. Smith. 1984. A short amino acid sequence able to specify nuclear location. *Cell* **39**: 499-509.

Kalinich, J.F., & M.G. Douglas. 1989. *In vitro* translocation through the yeast nuclear envelope: signal-dependent transport requires ATP and calcium. *J. Biol. Chem.* **264**: 17979-17989.

Kambach, C., & I.W. Mattaj. 1992. Intracellular distribution of the U1A protein depends on active transport and nuclear binding to U1 snRNA. *J. Cell Biol.* **118**: 11-21.

Kambach, C., & I.W. Mattaj. 1994. Nuclear transport of the U2 snRNP-specific U2B″ protein is mediated by both direct and indirect signalling mechanisms. *J. Cell Sci.* **107**: 1807-1816.

Kemler, I., G. Whittaker, & A. Helenius. 1994. Nuclear import of microinjected influenza virus ribonucleoproteins. *Virol.* **202**: 1028-1033.

Khanna-Gupta, A., & V.C. Ware. 1989. Nucleocytoplasmic transport of ribosomes in a eukaryotic system: is there a facilitated transport process? *Proc. Natl. Acad. Sci. USA* **86**: 1791-1795.

Kleinschmidt, J.A., & A. Seiter. 1988. Identification of domains involved in nuclear uptake and histone binding of protein N1 of *Xenopus laevis*. *EMBO J.* **7**: 1605-1614.

Kraemer, D., R.W. Wozniak, G. Blobel, & A. Radu. 1994. The human CAN protein, a putative oncogene product associated with myeloid leukemogenesis, is a nuclear pore complex protein that faces the cytoplasm. *Proc. Natl. Acad. Sci. USA* **91**: 1519-1523.

Kraemer, D.M., C. Strambio-de-Castillia, G. Blobel, & M.P. Rout. 1995. The essential yeast nucleoporin NUP159 is located on the cytoplasmic side of the nuclear pore complex and serves in karyopherin-mediated binding of transport substrate. *J. Biol. Chem.* **270**: 19017-19021.

LaCasse, E.C., & Y.A. Lefebvre. 1995. Nuclear localization signals overlap DNA- or RNA-binding domains in nucleic acid binding. *Nucleic Acids Res.* **23**: 1647-1656.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**: 680-685.

Lagaye, S., J-P. Barque, M. le Maire, H. Denis, & C-J. Larsen. 1988. Characterization by human autoantibodies of two HeLa cell proteins which are related to *Xenopus laevis* transcription factor TFIIIA. *Nucleic Acids Res.* **16**: 2473-2487.

Lanford, R.E., P. Kanda, & R.C. Kennedy. 1984. Induction of nuclear transport with a synthetic peptide homologous to the SV40 T-antigen transport signal. *Cell* **46**: 575-582.

Lanford, R.E., C.M. Feldherr, R.G. White, R.G. Dunham, & P. Kanda. 1990. Comparison of diverse transport signals in synthetic peptide-induced nuclear transport. *Exp. Cell Res.* **186**: 32-38.

Laskey, R.A., & C. Dingwall. 1993. Nuclear shuttling: The default pathway for nuclear proteins? *Cell* **74**: 585-586.

Lee, W-C., & T. Mélèse. 1989. Identification and characterization of a nuclear localization sequence-binding protein in yeast. *Proc. Natl. Acad. Sci. USA* **86**: 8808-8812.

Lee, W.C., Z. Xue, & T. Mélèse. 1991. The NSR1 gene encodes a protein that specifically binds nuclear localization sequences and has two RNA recognition motifs. *J. Cell Biol.* **113**: 1-12.

Lewis, P., & M.E. Emerman. 1994. Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J. Virol.* **68**: 510-516.

Li, R., & J.O. Thomas. 1989. Identification of a human protein that interacts with nuclear localization signals. *J. Cell Biol.* **109**: 2623-2632.

Lührmann, R., B. Kastner, & M. Bach. 1990. Structure of spliceosomal snRNPs and their role in pre-mRNA splicing. *Biochim. Biophys. Acta* **1087**: 265-292.

Mairy, M., & H. Denis. 1971. Recherches biochimiques sur l'oogenèse. 1. Synthèse et accumulation du RNA pendant l'oogenèse du crapaud Sud-African *Xenopus laevis*. *Devel. Biol.* **24**: 143-165.

Mandell, R.B., & C.M. Feldherr. 1990. Identification of two HSP70-related *Xenopus* oocyte proteins that are capable of recycling across the nuclear envelope. *J. Cell Biol.* **111**: 1775-1783.

Martin, K., & A. Helenius. 1991a. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. *Cell* **67**: 117-130.

Martin, K., & A. Helenius. 1991b. Transport of incoming influenza virus nucleocapsids into the nucleus. *J. Virol.* **65**: 232-244.

Marshallsay, C., & R. Lührmann. 1994. *In vitro* nuclear import of snRNPs: cytosolic factors mediate m_3G-cap dependence of U1 and U2 snRNP transport. *EMBO J.* **13**: 222-231.

Mattaj, I.W., S. Lienhard, R. Zeller, & E.M. DeRobertis. 1983. Nuclear exclusion of transcription factor IIIA and the 42S particle transfer RNA-binding protein in *Xenopus* oocytes: a possible mechanism for gene control? *J. Cell Biol.* **97**: 1261-1265.

Maxwell, E.S., & M.J. Fournier. 1995. The small nucleolar RNAs. *Annu. Rev. Biochem.* **35**: 897-934.

Mazzanti, M., B. Innocenti, & M. Rigatelli. 1994. ATP-dependent ionic permeability of nuclear envelope in *in situ* nuclei of *Xenopus* oocytes. *FASEB J.* **8**: 231-236.

Mehlin, H., B. Daneholt, & U. Skoglund. 1992. Translocation of a specific premessenger ribonucleoprotein particle through the nuclear pore studied with electron microscope tomography. *Cell* **69**: 605-613.

Mehlin, H., B. Daneholt, & U. Skoglund. 1995. Structural interaction between the nuclear pore complex and a specific translocating RNP particle. *J. Cell Biol.* **129**: 1205-1216.

Meier, U.T., & G. Blobel. 1990. A nuclear localization signal binding protein in the nucleolus. *J. Cell Biol.* **111**: 2235-2245.

Meier, U.T., & G. Blobel. 1992. Nopp140 shuttles on tracks between nucleolus and cytoplasm. *Cell* **70**: 127-138.

Melchior, F., B. Paschal, J. Evans, & L. Gerace. 1993. Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. *J. Cell Biol.* **123**: 1649-1659.

Meßmer, B., & C. Dreyer. 1993. Requirements for nuclear translocation and nucleolar accumulation of nucleolin of *Xenopus laevis*. *Eur. J. Cell Biol.* **61**: 369-382.

Meyer, B.E., & M.H. Malim. 1994. The HIV-1 Rev *trans*-activator shuttles between the nucleus and the cytoplasm. *Genes Devel.* **8**: 1538-1547.

Michaud, N., & D.S. Goldfarb. 1991. Multiple pathways in nuclear transport: the import of U2 snRNP occurs by a novel kinetic pathway. *J. Cell Biol.* **112**: 215-223.

Michaud, N., & D.S. Goldfarb. 1992. Microinjected U snRNAs are imported to oocyte nuclei via the nuclear pore complex by three distinguishable targeting pathways. *J. Cell Biol.* **116**: 851-861.

Michaud, N., & D.S. Goldfarb. 1993. Most nuclear proteins are imported by a single pathway. *Exp. Cell Res.* **208**: 128-136.

Moll, T., G. Tebb, U. Surana, H. Robitsch, & K. Nasmyth. 1991. The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the *S. cerevisiae* transcription factor SW15. *Cell* **66**: 743-758.

Moore, M.S., & G. Blobel. 1992. The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. *Cell* **69**: 939-950.

Moore, M.S., & G. Blobel. 1993. The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature* **365**: 661-663.

Moore, M.S., & G. Blobel. 1994a. A G protein involved in nucleocytoplasmic transport: the role of Ran. *Trends Biochem. Sci.* **19**: 211-216.

Moore, M.S., & G. Blobel. 1994b. Purification of a Ran-interacting protein that is required for protein import into the nucleus. *Proc. Natl. Acad. Sci. USA* **91**: 10212-10216.

Moreland, R.B., H.G. Nam, L.M. Hereford, & H.M. Fried. 1985. Identification of a nuclear localization signal of a yeast ribosomal protein. *Proc. Natl. Acad. Sci. USA* **82**: 6561-6565.

Moroianu, J., & G. Blobel. 1995. Protein export from the nucleus requires the GTPase Ran and GTP hydrolysis. *Proc. Natl. Acad. Sci. USA* **92**: 4318-4322.

Moroianu, J., G. Blobel, & A. Radu. 1995a. A previously identified protein of uncertain function is karypherin-α and together with karyopherin-β docks import substrate at nuclear pore complexes. *Proc. Natl. Acad. Sci. USA* **92**: 2088-2091.

Moroianu, J., M. Hijikata, G. Blobel, & A. Radu. 1995b. Mammalian karyopherin α_1B and α_2B heterodimers: α_1 or α_2 subunit binds nuclear localization signal and β subunit interacts with peptide repeat-containing nucleoporins. *Proc. Natl. Acad. Sci. USA* **92**: 6532-6536.

Nelson, M., & P. Silver. 1989. Context affects nuclear protein localization in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **9**: 384-389.

Neuman de Vegvar, H.E. & J.E. Dahlberg. 1990. Nucleocytoplasmic transport and processing of small nuclear RNA precursors. *Mol. Cell. Biol.* **10**: 3365-3375.

Newmeyer, D.D., & D.J. Forbes. 1988. Nuclear import can be separated into distinct steps in vitro: nuclear pore binding and translocation. *Cell* **52**: 641-653.

Newmeyer, D.D., & D.J. Forbes. 1990. An *N*-ethylmaleimide-sensitive cytosolic factor necessary for nuclear protein import: requirement in signal-mediated binding to the nuclear pore. *J. Cell Biol.* **110**: 547-557.

Newmeyer, D.D., D.R. Finlay, & D.J. Forbes. 1986a. In vitro transport of a fluorescent nuclear protein and exclusion of non-nuclear proteins. *J. Cell Biol.* **103**: 2091-2102.

Newmeyer, D.D., J.M. Lucocq, T.R. Bürglin, & E.M. De Robertis. 1986b. Assembly *in vitro* of nuclei active in nuclear protein transport: ATP is required for nucleoplasmin accumulation. *EMBO J.* **5**: 501-510.

Nigg, E.A., P.A. Baeuerle, & R. Lührmann. 1991. Nuclear import-export: in search of signals and mechanisms. *Cell* **66**: 15-22.

Nirmala, P.B., & R.V. Thampan. 1995a. A 55-kDa protein (p55) of the goat uterus mediates nuclear transport of the estrogen receptor. 1. Purification and characterization. *Arch. Biochem. Biophys.* **319**: 551-561.

Nirmala, P.B., & R.V. Thampan. 1995b. A 55-kDa protein (p55) of the goat uterus mediates transport of the estrogen receptor. 2. Details of the transport mechanism. *Arch. Biochem. Biophys.* **319**: 562-569.

Ogata, K., A. Kurahashi, C. Nishiyama, & K. Terao. 1993. Metabolic characteristics of rat liver cytosolic 5S RNA. *Eur. J. Biochem.* **213**: 1277-1282.

Okuno, Y., N. Imamoto, & Y. Yoneda. 1993. 70-kDa heat-shock protein colocalizes with karyophilic proteins into the nucleus during their transport *in vitro*. *Exp. Cell Res.* **206**: 134-142.

Paine, P.L. 1992. Diffusion between nucleus and cytoplasm. In: **Feldherr, C.M.** (ed). *Nuclear Trafficking*. San Diego, Academic Press: 3-14.

Paine, P.L. 1993. Nuclear protein accumulation by facilitated transport and intranuclear binding. *Trends Cell Biol.* **3**: 325-329.

Paine, P.L., R. Yassin, T.M. Paine, I. Vancurova, C. George, & N. Sahay. 1995. Intranuclear binding of nucleoplasmin. *J. Cell. Biochem.* **58**: 105-114.

Panté, N, & U. Aebi. 1993. The nuclear pore complex. *J. Cell Biol.* **122**: 977-984.

Panté, N, & U. Aebi. 1994. Towards the molecular details of the nuclear pore complex. *J. Struct. Biol.* **113**: 179-189.

Park, M.K., M. D'Onofrio, M.C. Willingham, J.A. Hanover. 1987. A monoclonal antibody against a family of nuclear pore proteins (nucleoporins): O-linked *N*-acetylglucosamine is part of the immunodeterminant. *Proc. Natl. Acad. Sci. USA* **84**: 6462-6466.

Parry, H.D., D. Scherly, & I.W. Mattaj. 1989. 'Snurpogenesis': the transcription and assembly of U snRNP components. *Trends Biochem. Sci.* **14**: 15-19.

Paschal, B.M. & L. Gerace 1995. Identification of NTF2, a cytosolic factor for nuclear import that interacts with nuclear pore complex protein p62. *J. Cell Biol.* **129**: 925-937.

Pelham, H.R.B. & D.D. Brown. 1980. A specific transcription factor that can bind either the 5S RNA gene or 5S RNA. *Proc. Natl. Acad. Sci. USA* **77**: 4170-4174.

Peppel, K., & C. Baglioni. 1990. A simple and fast method to extract RNA from tissue culture cells. *Biotechniques* **9**: 711-713.

Peters, R. 1984. Nucleo-cytoplasmic flux and intracellular mobility in single hepatocytes measured by fluorescence microphotolysis. *EMBO J.* **3**: 1831-1836.

Peters, R. 1986. Fluorescence microphotolysis to measure nucleocytoplasmic transport and intracellular mobility. *Biochim. Biophys. Acta* **864**:305-359.

Picard, B., & M. Wegnez. 1979. Isolation of a 7S particle from *Xenopus laevis* oocytes: a 5S RNA-protein complex. *Proc. Natl. Acad. Sci. USA* **76**: 241-245.

Picard, B., M. le Maire, M. Wegnez, & H. Denis. 1980. Biochemical research on oogenesis. Composition of the 42-S storage particles of *Xenopus laevis* oocytes. *Eur. J. Biochem.* **109**: 359-368.

Piñol-Roma, S., & G. Dreyfuss. 1992. Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. *Nature* **355**: 730-732.

Pippin, J., V. Contractor, & J.L. Ambrus. 1994. The use of signal-distinguishable probes in differential or sequential autoradiography in hybridization analysis. *Biotechniques* **16**: 680-682.

Pokrywka, N.J., & D.S. Goldfarb. 1995. Nuclear export pathways of tRNA and 40S ribosomes include both common and specific intermediates. *J. Biol. Chem.* **270**: 3619-3624.

Powers, M.A., & D.J. Forbes. 1994. Cytosolic factors in nuclear transport: what's importin? *Cell* **79**: 931-934.

Pruschy, M., Y. Ju, L. Spitz, E. Carafoli, & D.S. Goldfarb. 1994. Facilitated nuclear transport of calmodulin in tissue culture cells. *J. Cell Biol.* **6**: 1527-1536.

Radu, A., G. Blobel, & M.S. Moore. 1995a. Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrates to distinct nucleoporins. *Proc. Natl. Acad. Sci. USA* **92**: 1769-1773.

Radu, A., M.S. Moore, & G. Blobel. 1995b. The peptide repeat domain of nucleoporin functions as a docking site in transport across the nuclear pore complex. *Cell* **81**: 215-222.

Raška, I., R.L. Ochs, & L. Salamin-Michel. 1990. Immunocytochemistry of the cell nucleus. *Electron Microsc. Rev.* **3**: 301-353.

Raška, I., M. Dundr, K. Koberna, I. Melčák, M-C. Risueño, & I. Török. 1995. Does synthesis of ribosomal RNA take place within nucleolar fibrillar centres or dense fibrillar components? A critical appraisal. *J. Struct. Biol.* **114**: 1-22.

Richardson, W.D., A.D. Mills, S.M. Dillworth, R.A. Laskey, & C. Dingwall. 1988. Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. *Cell* **52**: 655-664.

Richter, J.D., & D. Standiford. 1992. Structure and regulation of nuclear localization signals. In: **Feldherr, C.M.** (ed). *Nuclear Trafficking*. San Diego, Academic Press: 89-120.

Rihs, H., & R. Peters. 1989. Nuclear transport kinetics depend on phosphorylation site-containing sequences flanking the karyophilic signal of the simian virus 40 T-antigen. *EMBO J.* **8**: 1479-1484.

Rihs, H., D.A. Jans, H. Fan, & R. Peters. 1991. The rate of nuclear cytoplasmic protein transport is determined by the casein kinase II site flanking the nuclear localization sequence of the SV40 T-antigen. *EMBO J.* **10**: 633-639.

Ris, H. 1991. The 3-D structure of the nuclear pore complex as seen by high voltage electron microscopy and high resolution low voltage scanning electron microscopy. *EMSA Bull.* **21**: 54-56.

Robbins, J., S.M. Dilworth, R.A. Laskey, & C. Dingwall. 1991. Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* **64**: 615-623.

Roberts, B. 1989. Nuclear location signal-mediated protein transport. *Biochim. Biophys. Acta* **1008**:263-280.

Roberts, B.L., W.D. Richardson, & A.E. Smith. 1987. The effect of protein context on nuclear location signal function. *Cell* **50**: 465-475.

Roe, T., T.C. Reynolds, G. Yu, & P.O. Brown. 1993. Integration of murine leukaemia virus DNA depends on mitosis. *EMBO J.* **12**: 2099-2108.

Romac, J. M-J., D.H. Graff, & J.D. Keene. 1994. The U1 small nuclear ribonucleoprotein (snRNP) 70K protein is transported independently of U1 snRNP particles via a nuclear localization signal in the RNA-binding domain. *Mol. Cell. Biol.* **14**: 4662-4670.

Romaniuk, P.J., I. Leal de Stevenson, & H. H-A. Wong. 1987. Defining the binding site of *Xenopus* transcription factor IIIA on 5S RNA using truncated and chimeric 5S RNA molecules. *Nucleic Acids Res.* **15**: 2737-2755.

Rosbash, M., & R.H. Singer. 1993. RNA travel: tracks from DNA to cytoplasm. *Cell* **75**: 399-401.

Rout, M.P., & S.R. Wente. 1994. Pores for thought: nuclear pore complex proteins. *Trends Biochem. Sci.* **4**: 357-365.

Saitoh. H., & M. Dasso. 1995. The RCC1 protein interacts with Ran, RanBP1, hsc70, and a 340-kDa protein in *Xenopus* extracts. *J. Biol. Chem.* **270**: 10658-10663.

Savino, R., Y. Hitti, & S.A. Gerbi. 1992. Genes for *Xenopus laevis* U3 small nuclear RNA. *Nucleic Acids Res.* **20**: 5434-5442.

Schaap, P.J., J. van't Riet, C.L. Woldringh, & H.A. Raué. 1991. Identification and functional analysis of the nuclear localization signals of ribosomal protein L25 from *Saccharomyces cerevisiae*. *J. Mol. Biol.* **221**: 225-237.

Schlenstedt, G., C. Saavendra, J.D.J. Loeb, C.N. Cole, & P.A. Silver. 1995. The GTP-bound form of the yeast Ran/TC4 homologue blocks nuclear protein import and appearance of poly(A)$^+$ RNA in the cytoplasm. *Proc. Natl. Acad. Sci. USA* **92**: 225-229.

Schliephake, A.W., & A. Rethwilm. 1994. Nuclear localization of foamy virus gag precursor protein. *J. Virol.* **68**: 4946-4954.

Schmidt-Zachmann, M.S., & E.A. Nigg. 1993. Protein localization to the nucleolus: a search for targeting domains in nucleolin. *J. Cell Sci.* **105**: 799-806.

Schmidt-Zachmann, M.S., B. Hügle-Dörr, & W.W. Franke. 1987. A constitutive nucleolar protein identified as a member of the nucleoplasmin family. *EMBO J.* **6**: 1881-1890.

Schmidt-Zachmann, M.S., C. Dargemont, L.C. Kühn, & E.A. Nigg. 1993. Nuclear export of proteins: the role of nuclear retention. *Cell* **74**: 493-504.

Schröder, H.C., M. Rottmann, M. Bachmann, & W.E.G. Müller. 1986a. Purification and characterization of the major nucleoside triphosphatase from rat liver nuclear envelopes. *J. Biol. Chem.* **261**: 663-668.

Schröder, H.C., M. Rottmann, M.Bachmann, W.E.G. Müller, A.R. McDonald, & P.S. Agutter. 1986b. Proteins from rat liver cytosol which stimulate mRNA transport. *Eur. J. Biochem.* **159**: 51-59.

Schröder, H.C., R. Becker, M. Bachmann, M. Gramzow, A-P. Sève, M. Monsigny, & W.E.G. Müller. 1986c. Differential changes of nuclear-envelope-associated enzyme activities involved in nucleocytoplasmic mRNA transport in the developing rat brain and liver. *Biochim. Biophys. Acta* **868**: 108-118.

Schröder, H.C., M. Rottmann, R. Wegner, M. Bachmann, A. Dorn, & W.E.G. Müller. 1988. Studies on protein kinases involved in regulation of nucleocytoplasmic mRNA transport. *Biochem. J.* **252**: 777-790.

Schröder, H.C., D. Ugarkovic, P. Langen, M. Bachmann, A. Dorn, Y. Kuchino, & W.E.G. Müller. 1990. Evidence for involvement of a nuclear envelope-associated RNA helicase activity in nucleocytoplasmic RNA transport. *J. Cell. Physiol.* **145**: 136-146.

Shaulsky, G., N. Goldfinger, A. Ben-Ze'ev, & V. Rotter. 1990. Nuclear accumulation of p53 protein is mediated by several nuclear localization signals and plays a role in tumorigenesis. *Mol. Cell. Biol.* **10**: 6565-6577.

Shi, Y., & J.O. Thomas. 1992. The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. *Mol. Cell. Biol.* **12**: 2186-2192.

Silver, P.A. 1991. How proteins enter the nucleus. *Cell* **64**: 489-497.

Silver, P., & H. Goodson. 1989. Nuclear protein transport. *Crit. Rev. Biochem. Mol. Biol.* **24**: 419-435.

Silver, P.A., A. Chiang, & I. Sadler. 1988. Mutations that alter both localization and production of a yeast nuclear protein. *Genes Dev.* **2**: 707-717.

Simons, F.H.M., G. J.M. Prujin, & W.J. van Venrooij. 1994. Analysis of the intracellular localization and assembly of Ro ribonucleoprotein particles by microinjection into *Xenopus laevis* oocytes. *J. Cell Biol.* **125**: 981-988.

Simos, G., & E.C. Hurt. 1995. Nucleocytoplasmic transport: factors and mechanisms. *FEBS Letters.* **369**: 107-112.

Singh, R., & M.R. Green. 1993. Sequence-specific binding of transfer RNA by glyceraldehyde-3-phosphate dehydrogenase. *Science* **259**: 365-368.

Siomi, H., & G. Dreyfuss. 1995. A nuclear localization domain in the hnRNP A1 protein. *J. Cell Biol.* **129**: 551-560.

Snow, C.M., A. Senior, & L. Gerace. 1987. Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. *J. Cell Biol.* **104**: 1143-1156.

Standiford, D.M., & J.D. Richter. 1992. Analysis of a developmentally regulated nuclear localization signal in *Xenopus*. *J. Cell Biol.* **118**: 991-1002.

Starr, C.M., & J.A. Hanover. 1990. Glycosylation of nuclear pore protein p62. *J. Biol. Chem.* **265**: 6868-6873.

Steitz, J.A., C.Berg, J.P.Hendrick, H. La Branche-Chabot, A. Metspalu, J. Rinke, & T. Yario. 1988. A 5S rRNA/L5 complex is a precursor to ribosome assembly in mammalian cells. *J. Cell Biol.* **106**: 545-556.

Sterne-Marr, R., J.M. Blevitt, & L. Gerace. 1992. O-linked glycoproteins of the nuclear pore complex interact with a cytosolic factor required for nuclear protein import. *J. Cell Biol.* **116**: 271-280.

Stevenson, M. 1994. Identification of factors that govern HIV-1 replication in nondividing host cells. *Aids Res. Human Retroviruses* **10**: S11-S15.

Stochaj, U., & P.A. Silver. 1992. Nucleocytoplasmic transport of proteins. *Eur. J. Cell Biol.* **59**: 1-11.

Stutz, F., M. Nelville, & M. Rosbash. 1995. Identification of a novel nuclear pore-associated protein as a functional target of the HIV-1 Rev protein in yeast. *Cell* **82**: 495-506.

Sukegawa, J., & G. Blobel. 1993. A nuclear pore complex protein that contains zinc finger motifs, binds DNA and faces the nucleoplasm. *Cell* **72**: 29-38.

Szebeni, A., J.E. Herrera, & M.O.J. Olson. 1995. Interaction of nucleolar protein B23 with peptides related to nuclear localization signals. *Biochemistry* **34**: 8037-8042.

Tartakoff, A.M., & R. Schneiter. 1995. The nuclear GTPase cycle: promoting peripheralization? *Trends Cell Biol.* **5**: 5-8.

Terns, M., & J.E. Dahlberg. 1994. Retention and 5′ cap trimethylation of U3 snRNA in the nucleus. *Science* **264**: 959-961.

Terns, M.P., J.E. Dahlberg, & E. Lund. 1993. Multiple *cis*-acting signals for export of pre-U1 snRNA from the nucleus. *Genes Devel.* **7**: 1898-1908.

Terns, M.P., C. Grimm, E. Lund, & J.E. Dahlberg. 1995. A common maturation pathway for small nucleolar RNAs. *EMBO J.* **14**: 4860-4871.

Tobian, J.A., L. Drinkard, & M. Zasloff. 1985. tRNA nuclear transport: defining the critical regions of human $tRNA^{met}_i$ by point mutagenesis. *Cell* **43**: 415-422.

Underwood, M.R., & H.M. Fried. 1990. Characterization of nuclear localization sequences derived from yeast ribosomal protein L29. *EMBO J.* **9**: 91-99.

Van Zee, K., A. Dickmans, U. Fischer, R. Lührmann, & E. Fanning. 1993. A cytoplasmically anchored nuclear protein interferes specifically with the import of nuclear proteins but not U1 snRNA. *J. Cell Biol.* **121**: 229-240.

Vancurova, I., W. Lou, T.M. Paine, & P.L. Paine. 1993. Nucleoplasmin uptake by facilitated transport and intranuclear binding. *Eur. J. Cell Biol.* **62**: 22-33.

Vancurova, I., J. Jochova, W. Lou, & P.L. Paine. 1994. An NLS is sufficient to engage facilitated translocation by the nuclear pore complex and subsequent intranuclear binding. *Biochem. Biophys. Res. Comm.* **205**: 529-536.

Vancurova, I., T. Maloney Paine, W. Lou, & P.L. Paine. 1995. Nucleoplasmin associates with and is phosphorylated by casein kinase II. *J. Cell Sci.* **108**: 779-787.

Varagona, M.J., R.J. Schmidt, N.V. Raikhel. 1992. Nuclear localization signal(s) required for nuclear targeting of the maize regulatory protein opaque-2. *Plant Cell* **4**: 1213-1227.

Wagner, P., J. Kunz, A. Koller, & M.N. Hall. 1990. Active transport of proteins into the nucleus. *FEBS Lett.* **275**: 1-5.

Walter, P., & V.R. Lingappa. 1986. Mechanism of protein translocation across the endoplasmic reticulum membrane. *Annu. Rev. Cell Biol.* **2**: 499-516.

Ware, V.C., & A. Khanna-Gupta. 1990. Nucleocytoplasmic transport of ribosomal subunits. In: **Hill, W.E., A. Dahlberg, R.A. Garrett, P.B. Moore, D. Schlessinger, & J.R. Warner** (eds), *The Ribosome: Structure, Function, and Evolution.* Washington DC, American Society for Microbiology: 470-476.

Wegnez, M., R. Monier, & H. Denis. 1972. Sequence heterogeneity of 5S RNA in *Xenopus laevis*. *FEBS Lett.* **25**: 13-20.

Weis, K., I. Mattaj, & A. Lamond. 1995. Identification of hSRP1α as a functional receptor for nuclear localization sequences. *Science* **268**: 1049-1053.

Wen. W., J.L. Meinkoth, R.Y. Tsien, & S.S. Taylor. 1995. Identification of a signal for rapid export of proteins from the nucleus. *Cell* **82**: 463-473.

Wickens, M.P., & J.E. Dahlberg. 1987. RNA-protein interactions. *Cell* **51**: 339-342.

Wilkin, N., U. Kosser, J-L. Senécal, U. Scheer, & M-C. Dabauvalle. 1993. Nup180, a novel nuclear pore complex protein localizing to the cytoplasmic ring and associated fibrils. *J. Cell Biol.* **123**: 1345-1354.

Williams, A.S., T.C. Ingledue III, B.K. Kay, & W.F. Marzluff. 1994. Changes in the stem-loop at the 3′ terminus of histone mRNA affects its nucleocytoplasmic transport and cytoplasmic regulation. *Nucleic Acids Res.* **22**: 4660-4666.

Wolff, B., M.C. Willingham, & J.A. Hanover. 1988. Nuclear protein import: specificity for transport across the nuclear pore. *Exp. Cell Res.* **178**:318-334.

Wolffe, A.P., & D.D. Brown. 1988. Developmental regulation of two 5S ribosomal RNA genes. *Science* **241**: 1626-1632.

Wormington, W.M. 1989. Developmental expression and 5S rRNA-binding activity of *Xenopus laevis* ribosomal protein L5. *Mol. Cell. Biol.* **9**: 5281-5288.

Wormington, W.M., & D.D. Brown. 1983. Onset of 5S RNA gene regulation during *Xenopus* embryogenesis. *Devel. Biol.* **99**: 248-257.

Wu, J., M.J. Matunis, D. Kraemer, G. Blobel, & E. Coutavas. 1995a. Nup358, a cyoplasmically exposed nucleoporin with peptide repeats, Ran-GTP binding sites, zinc fingers, a cyclophilin a homologous domain, and a leucine-rich region. *J. Biol. Chem.* **270**: 14209-14213.

Wu, M-H., C-Y. Lam, & B.Y-M. Yung. 1995b. Translocation of nucleophosmin from nucleoli to nucleoplasm requires ATP. *Biochem. J.* **305**: 987-992.

Xue, Z., & T. Mélèse. 1994. Nucleolar proteins that bind NLSs: a role in nuclear import or ribosome biogenesis? *Trends Cell Biol.* **4**: 414-417.

Xue, Z., X. Shan, B. Lapeyre, & T. Mélèse. 1993. The amino terminus of mammalian nucleolin specifically recognises SV40 T-antigen type nuclear localization sequences. *Eur. J. Cell Biol.* **62**: 13-21.

Yamada, M, & H. Kasamatsu. 1993. Role of the nuclear pore complex in simian virus 40 nuclear targeting. *J. Virol.* **67**: 119-130.

Yang, J., & D.B. DeFranco. 1994. Differential roles of heat shock protein 70 in the in vitro nuclear import of glucocorticoid receptor and simian virus 40 large tumor antigen. *Mol. Cell Biol.* **14**: 5088-5098.

Yeh, C-T., Y-F. Liaw, & J-H. Ou. 1990. The arginine-rich domain of hepatitis B virus precore and core proteins contains a signal for nuclear transport. *J. Virol.* **64**: 6141-6147.

Yeh, L.C.C., & J.C. Lee. 1995. An *in vitro* system for studying RNA-protein interaction: application to a study of yeast ribosomal protein L1 binding to 5S rRNA. *Biochimie* **77**: 167-173.

Ylikomi, T., M.T. Bocquel, M. Berry, H. Gronemeyer, & P. Chambon. 1992. Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. *EMBO J.* **11**: 3681-3694.

Yokoyama, N., N. Hayashi, T. Seki, N. Panté, T. Ohba, K. Nishii, K. Kuma, T. Itayashida, T. Miyata, U. Aebi, M. Fukui, & T. Nishimoto. 1995. A giant nucleopore protein that binds Ran/TC4. *Nature* **376**: 184-188.

Yoneda, Y., N. Imamoto-Sonobe, M. Yamaizumi & T. Uchida. 1987. Reversible inhibition of protein import into the nucleus by wheat germ agglutinin injected into cultured cells. *Exp. Cell Res.* **173**: 586-595.

Zasloff, M. 1983. tRNA transport from the nucleus in a eukaryotic cell: carrier-mediated translocation process. *Proc. Natl. Acad. Sci. USA* **80**: 6436-6440.

Zasloff, M., M. Rosenberg, & T. Santos. 1982. Impaired nuclear transport of a human variant of $tRNA^{Met}_{i}$. *Nature* **300**: 81-84.

Appendix

This reprint from *Molecular and Cellular Biology* contains results from part of the work presented in this thesis. My contribution to this publication consisted of the studies investigating the nuclear import of oocyte-type and the two mutant 5S rRNAs, 41-44 nt and 96-101 nt, with respect to temperature-dependence, ATP requirement and the effect of wheat germ agglutinin.

Signed: K.J. Mundwil

Date: November 29, 1995

MOLECULAR AND CELLULAR BIOLOGY, Nov. 1993, p. 6819–6831 Vol. 13, No. 11
0270-7306/93/116819-13$02.00/0

Structural Requirements of 5S rRNA for Nuclear Transport, 7S Ribonucleoprotein Particle Assembly, and 60S Ribosomal Subunit Assembly in *Xenopus* Oocytes

LIZABETH A. ALLISON,[1]* MELANIE T. NORTH,[1] KIRSTIE J. MURDOCH,[1] PAUL J. ROMANIUK,[2] STÉPHANE DESCHAMPS,[3]† AND MARC LE MAIRE[3]†

Department of Zoology, University of Canterbury, Christchurch, New Zealand[1]; Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia V8W 3P6, Canada[2]; and Centre de Génétique Moléculaire, Laboratoire propre du Centre National de la Recherche Scientifique, associé à l'Université P. et M. Curie (Paris VI), F-91198 Gif-sur-Yvette Cedex, France[3]

Received 4 May 1993/Returned for modification 7 July 1993/Accepted 18 August 1993

Structural requirements of 5S rRNA for nuclear transport and RNA-protein interactions have been studied by analyzing the behavior of oocyte-type 5S rRNA and of 31 different in vitro-generated mutant transcripts after microinjection into the cytoplasm of *Xenopus* oocytes. Experiments reveal that the sequence and secondary and/or tertiary structure requirements of 5S rRNA for nuclear transport, storage in the cytoplasm as 7S ribonucleoprotein particles, and assembly into 60S ribosomal subunits are complex and nonidentical. Elements of loops A, C, and E, helices II and V, and bulged and hinge nucleotides in the central domain of 5S rRNA carry the essential information for these functional activities. Assembly of microinjected 5S rRNA into 60S ribosomal subunits was shown to occur in the nucleus; thus, the first requirement for subunit assembly is nuclear targeting. The inhibitory effects of ATP depletion, wheat germ agglutinin, and chilling on the nuclear import of 5S rRNA indicate that it crosses the nuclear envelope through the nuclear pore complex by a pathway similar to that used by karyophilic proteins.

The orchestration of ribosome biogenesis in eukaryotic cells is a process that requires transfer of macromolecules into and out of the nucleus. In *Xenopus* oocytes, 5S rRNA is shuttled between the nuclear and cytoplasmic compartments of the oocyte during different stages of oogenesis in a complex pathway involving different protein associations. In previtellogenic oocytes, 5S rRNA is synthesized before other components of ribosomes are available, is exported from the nucleus, and stored in the cytoplasm as 7S ribonucleoprotein particles (RNPs) (5S rRNA complexed with transcription factor IIIA [TFIIIA]) or as 42S RNPs (5S rRNA complexed with other nonribosomal proteins and tRNA). During vitellogenesis, the 5S rRNA is released from storage and a 5S rRNA-ribosomal protein L5 complex, which is a precursor to assembly into the 60S large ribosomal subunit, forms (reference 3 and references therein). We are interested in the mechanisms that govern the subcellular trafficking of 5S rRNA within the oocyte, particularly the requirements for the mobilization of stored 5S rRNA during ribosome assembly.

There is ample evidence that transit of RNA and RNPs into and out of the nucleus occurs exclusively via the nuclear pores (20, 45; for a review, see reference 30). Although nuclear localization of proteins has been well characterized (1, 10, 21, 49; for a review, see reference 61), the factors governing nuclear export and import of RNA and RNPs remain enigmatic. Nuclear export of tRNA (75), pre-small nuclear RNAs (50), mRNA (16), 40S and 60S ribosomal subunits (6, 38), and 5S rRNA (24) occurs in a manner consistent with a mediated process. Analysis of the nuclear transport of U small nuclear RNAs has multiple defined, kinetically distinct targeting pathways (27, 47, 48). Nuclear transport of different classes of RNA may thus involve targeting to the pore complex by different cytoplasmic receptors and then translocation into the nucleus by the same pore complex-mediated mechanism. Specific RNA structures have been implicated as requirements for both nuclear import and export (5, 22, 32, 33, 67, 75). Translocation of RNA molecules across the nuclear envelope may also require interaction with specific proteins (28, 31, 33, 43, 62).

RNA-protein interactions are important for many regulatory processes. There is growing evidence that RNA structures, such as helices, loops, bulges, mismatches, and pseudoknots, are key elements in protein recognition (for a review, see reference 19). The sequence and structural requirements for binding of TFIIIA to 5S rRNA have been the subjects of extensive research (7, 8, 56, 66, 74). In comparison, the structural elements of the 5S rRNA molecule required for assembly into ribosomes and for its functional activity within the ribosome remain to be elucidated (34, 37, 69).

We report here the results of a structural analysis of 31 different 5S rRNA mutants by nuclear transport and RNP assembly assays in *Xenopus* oocytes. The results are discussed in relation to the abilities of these same 5S rRNA mutants to bind TFIIIA in vitro (7, 8, 56, 74). We show that determinants of nuclear transport, TFIIIA binding, and ribosome incorporation within the 5S rRNA molecule are complex and nonidentical. The results suggest that TFIIIA binding is not a prerequisite for nuclear targeting of 5S rRNA, consistent with earlier studies indicating that 7S RNPs are not imported (3, 44). We show that microinjected

* Corresponding author. Electronic mail address: l.allison@csc.canterbury.ac.nz.

† Present address: Section de Biophysique des Protéines et des Membranes, Département de Biologie Cellulaire et Moléculaire, Commissariat à l'Énergie Atomique et Centre National de la Recherche Scientifique URA 1290, CE Saclay, F-91191 Gif-sur-Yvette Cedex, France.

5S rRNA is incorporated into 60S ribosomal subunits in the nucleus; thus, the first requirement for assembly is nuclear import. Finally, we demonstrate that nuclear import of 5S rRNA is sensitive to general inhibitors of nuclear pore-mediated translocation.

MATERIALS AND METHODS

Synthesis of mutant 5S rRNAs. The 5S rRNA genes used in these experiments were constructed from a series of synthetic oligonucleotides that were subsequently introduced into pUC18 as previously described (56, 58). Internally labelled 5S rRNAs were produced by in vitro transcription from these gene templates with T7 RNA polymerase (Boehringer Mannheim N.Z. Ltd., Auckland, New Zealand) and 50 to 100 μCi of [α-^{32}P]GTP (3,000 Ci/mmol; Amersham Australia Pty Ltd., Auckland, New Zealand). The mixture was incubated for 1.5 h at 37°C and then treated with RNase-free DNase (Boehringer Mannheim). The sample was extracted with phenol and chloroform, and the RNA transcripts were precipitated twice with 2.5 M ammonium acetate and ethanol. The RNA pellet was resuspended in TE (10 mM Tris-HCl, 1 mM EDTA), pH 7.6, and stored at −80°C. RNA concentration was quantified by DNA Dipstick (Invitrogen Corp., San Diego, Calif.), according to the manufacturer's instructions.

Microinjection and analysis of nuclear transport. An ovary lobe was surgically removed from an adult *Xenopus laevis* (reared at the University of Canterbury on a diet of cockroaches), and the oocytes were separated by brief collagenase treatment as previously described (3). Stage 5-6 oocytes were microinjected with 40 to 80 nl of RNA (0.2 to 0.5 ng of RNA per oocyte) into the oocyte cytoplasm with a PV 830 PicoPump (World Precision Instruments, Inc., New Haven, Conn.) by previously published procedures (3). After overnight incubation (18 h) in O-R2 medium (3), nuclei were manually dissected from oocytes in 1% trichloroacetic acid and collected for analysis (3). RNA was extracted from nuclear and cytoplasmic fractions as described by Allison et al. (3). RNA was analyzed by 8% polyacrylamide–8 M urea gel electrophoresis as described previously (3). Dried gels were autoradiographed on Amersham Hyperfilm-MP at −80°C. A Kontron Uvikon 860 spectrophotometer equipped with a gel scan accessory was used to measure the intensity of bands on suitable exposures of autoradiograms (within the linear range of signal intensity of the film); data were quantified with software package 8543. The system has high resolution, and the software accommodates background levels and irregularly shaped bands. Only mutants that showed less than 50% nuclear transport relative to oocyte-type 5S rRNA were considered to be significantly impaired.

For ATP depletion assays, oocytes were preinjected with 50 nl of 1-U/μl apyrase (grade VIII; Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline (PBS) (3) to give a final intracellular concentration of 100 U/ml. Alternatively, 50 nl of PBS was preinjected as a control. After 30-min incubation at 18°C, oocytes were injected with labelled RNA. After an additional 6-h incubation, oocytes were analyzed for nuclear import as described above. ATP depletion was verified with a CLS ATP bioluminescence kit (Boehringer Mannheim). Single oocytes were homogenized in boiling 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.5, and incubated for 5 min at 100°C, and samples were then diluted 1:500 in double-distilled water. A 500-μl sample was added to an equal volume of luciferase extract immediately prior to measurement of luminescence with an SA1 ATP photometer (model 2000).

Wheat germ agglutinin (WGA) (Sigma Chemical Co.) was dissolved in PBS at concentrations ranging from 0.25 to 2 mg/ml. WGA (50 nl) or PBS (as a control) was injected into the oocyte cytoplasm. After incubation for 3 h, oocytes were injected with labelled RNA and analyzed for nuclear import as described above. As a control for the specificity of preinjection of 50 nl of 1-mg/ml WGA, 50 nl of 1-mg/ml WGA and 500 mM *N*-acetylglucosamine (Sigma Chemical Co.) were coinjected into oocytes prior to injection of labelled 5S rRNA. As a control for nonselective diffusion, oocytes were injected with 50 nl of 100-mg/ml fluorescein isothiocyanate-dextran (molecular weight, 10,000 or 150,000; Sigma Chemical Co.). After overnight incubation, nuclei were dissected out in nucleus isolation buffer (3) and viewed by fluorescence microscopy for diffusion of the dextran into the nucleus.

Since there can be variability in synthetic activity between different batches of oocytes, experiments were repeated a minimum of two times with oocytes from different animals.

Antisera. The antibodies raised against TFIIIA have been shown to react with the relevant protein; no cross-reaction with any other protein was noted by immunoblotting (70). Preparation of the antibodies raised against *Xenopus* 60S ribosomal subunits is described by Viel et al. (70); however, there is a slight difference in the reaction of the anti-60S ribosomal subunit antiserum used in this study (anti-serum number 2679; sampling date, 7 November 1985) and of the antiserum described by Viel et al. (70). Immunoblot analysis was performed on total protein from ribosomal subunits purified from mature *Xenopus* ovaries by ultracentrifugation techniques (70) or from 7S fractions obtained after sucrose density centrifugation of cell homogenates of *Xenopus* ovaries (41). The proteins were fractionated by polyacrylamide gel electrophoresis and either stained with Coomassie blue or transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore S.A., Saint-Quentin Yvelines, France). Membranes were incubated with diluted antiserum (1:287) in a solution of 10 mM sodium phosphate (pH 7.4), 150 mM NaCl, and 1% bovine serum albumin, washed in the same buffer, treated with ^{35}S-labelled-protein A in order to label the antigen-antibody complexes, washed again, and exposed to X-ray films at −80°C by previously published procedures (70).

To further ensure that the anti-60S antiserum did not cross-react with 7S RNPs, immunoprecipitation analyses were performed on 7S RNPs purified from immature *Xenopus* ovaries as previously described (3). Immunoprecipitation assays were performed as described by Allison et al. (3), using approximately 10 μg of 7S RNPs and 10 μl of antiserum bound to protein A-Sepharose (Pharmacia LKB Biotechnology, Auckland, New Zealand). Immunoprecipitates were vortexed briefly in sample buffer (2% sodium dodecyl sulfate, 100 mM dithiothreitol, 60 mM Tris [pH 6.8], 0.01% bromophenol blue), heated at 100°C for 5 min, and then centrifuged for 30 s. Proteins from the supernatant were resolved by polyacrylamide gel electrophoresis and silver stained with a Bio-Rad silver stain kit (Bio-Rad Laboratories Pty Ltd., Auckland, New Zealand), according to the manufacturer's instructions.

Immunoprecipitation assays. Microinjected oocytes were incubated for 48 h at 18°C. Immunoprecipitation assays were performed as described by Allison et al. (3), using homogenates of 20 microinjected oocytes and 10 μl of antiserum bound to protein A-Sepharose. For nuclear immunoprecipitations, 20 nuclei were isolated in nucleus isolation buffer

(3). RNA was recovered from the immunoprecipitates and immunosupernatants and resolved on 8% polyacrylamide–8 M urea gels, and then autoradiography was performed. Results were interpreted qualitatively by comparing the amounts of the different mutants immunoprecipitated with the amount of oocyte-type 5S rRNA immunoprecipitated and by comparing the relative specific activities of the RNA mutants and the amounts of RNA injected, using the supernatant fractions.

Electrophoretic mobility shift assays. After a 48-h incubation at 18°C, five microinjected oocytes were homogenized in 20 μl of RNP homogenization buffer (20 mM Tris [pH 7.6], 100 mM KCl, 1.5 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 U of RNasin per ml; Promega:Pacific Diagnostics Pty. Ltd., Auckland, New Zealand). A crude cellular lysate was prepared by disrupting oocytes with a Gilson tip, spinning down the cellular debris (yolk and pigment) by centrifugation at 9,000 × *g* for 10 min at 4°C, and removing the supernatant, taking care not to disturb the lipid pellicle floating on the surface. Glycerol-dye loading buffer was added to the supernatant, and samples were loaded directly onto a 6% polyacrylamide–0.1% Triton X-100 gel in Tris-borate-EDTA buffer plus 0.1% Triton X-100. Samples were electrophoresed at 300 V; gels were dried at 80°C for 30 min, and then autoradiography was performed. A sample of unlabelled, native 7S RNPs were included as a marker. The marker lane was stained with ethidium bromide and viewed by UV illumination.

RESULTS

Sequence and structural requirements for nuclear transport of 5S rRNA. The sequence and structural requirements of 5S rRNA for nuclear transport in *Xenopus* oocytes were investigated by analyzing 31 mutant 5S rRNA molecules generated by in vitro transcription. Figure 1 shows the secondary structure of oocyte-type 5S rRNA and the locations of the mutations analyzed. With the exception of seven mutants, substitutions were located between nucleotides 11 and 108 in the region of the RNA molecule shown to provide the necessary sequence and conformational information required for nuclear transport (3). In our previous study, however, we did not investigate quantitative differences in nuclear transport.

All mutant 5S rRNAs were stable 24 h after cytoplasmic microinjection (data not shown). Analysis of oocyte-type 5S rRNA revealed that on average, 14% of the microinjected RNA was found in the nucleus after 18 h (Fig. 2, lanes 1 and 2). These results were consistent with a previous study, which demonstrated that the total amount of labelled 5S rRNA found in the nucleus increases for up to 18 to 21 h and then remains constant (3). Presumably, this constant amount reflects steady-state levels of 5S rRNA molecules which have migrated into the nucleus and are being assembled into ribosomes. Nuclear transport of 5S rRNA thus represents a state of flux between the cytoplasm and nucleus, as opposed to a measure of nuclear import alone. The 31 mutant 5S rRNAs showed a variety of transport phenotypes. The intracellular distribution of five mutant RNAs is presented in Fig. 2. The most defective mutant, 16–21, showed a 75% reduction in nuclear transport relative to that of oocyte-type 5S rRNA (lanes 3 and 4). Mutants 41–44 and 105–108 were less defective in nuclear transport, showing reductions of 67 and 54%, respectively (compare lanes 5 and 6 and lanes 11 and 12). In contrast, mutants Δ63 and 67–70 had transport characteristics similar to those for oocyte-type 5S rRNA (lanes 7 to 10). The data are summarized in Table 1.

Mutations within predicted helices II and V tend to have lower nuclear transport values if the mutations are helix breaking (HB in Table 1), than if the mutations are helix maintaining (HM). Nucleotide substitutions that alter either the 5′ sequence of helix II or the sequence and structure of helix V resulted in a significant reduction (≥50%) in nuclear transport. The loop mutants tested have block sequence substitutions in one loop which alter the sequence but maintain the single-stranded secondary structure (56). Nucleotide substitutions that alter the 3′ sequence of loop C or the noncanonical base pairing in loop E (57, 72) resulted in a significant reduction in nuclear transport. The deletion of bulged nucleotides did not impair transport. In contrast, alteration of the hinge nucleotide at position 66 resulted in a 57% reduction in nuclear transport, suggesting this nucleotide is located in a key region of the 5S rRNA. This is in line with a previous study showing that nucleotides in the hinge region of the 5S rRNA, the junction of the three helical domains, play a central role in determining the coaxial stacking interactions and tertiary structure of the RNA and are critical for TFIIIA recognition (8). In summary, specific structural elements of the 5S rRNA molecule have been shown to be important for nuclear transport, but no single region of 5S rRNA is solely responsible.

TFIIIA has been implicated in mediating 5S rRNA nuclear export (31), but indirect evidence suggests that 7S RNPs are not import competent (3, 44). In order to determine whether there is a correlation between wild-type TFIIIA binding affinity and wild-type nuclear transport, results on nuclear transport of the mutants are compared with data on their ability to bind TFIIIA in vitro from previous studies (7, 8, 56, 74). In general, the two sets of data are comparable (Table 1); there are, however, important exceptions. For example, mutants 10–13, 57–62, and G109 possess low binding affinities for TFIIIA (17 to 40% of oocyte-type 5S rRNA), yet the measure of nuclear transport is closer to that of oocyte-type 5S rRNA (63 to 77%). Furthermore, other mutants (e.g., 96–101 and 14–15) with higher TFIIIA binding affinities (59 to 85%) exhibit defective nuclear transport (36 to 49%). Since some of the structural requirements of 5S rRNA for nuclear transport and 7S RNP assembly differ, this suggests that TFIIIA binding is not a prerequisite for nuclear targeting.

Nuclear import of 5S rRNA and mutant RNA molecules occurs by a mediated process. One possible explanation for oocyte-type and mutant 5S rRNAs entering the nucleus is that their small size allows for unrestricted entry. 5S rRNA is a 121-nucleotide molecule of approximately 41 kDa. The nuclear pore complex contains an aqueous channel of 9- to 11-nm diameter which acts like a molecular sieve; this channel allows rapid, nonselective diffusion of molecules of approximately 20 to 40 kDa, while excluding larger cytoplasmic proteins (52). A truncated, 98-nucleotide 5S rRNA does not enter the nucleus (Fig. 2) (3), however, which implies that nuclear uptake of full-length 5S rRNA occurs by a mechanism other than unrestricted entry. Further, comparing 5S rRNA and a globular protein may not be a valid comparison; the frictional ratio of naked 5S rRNA has been shown to be higher than that of 7S RNPs, suggesting that 5S rRNA is more elongated (17).

Translocation across the nuclear envelope occurs by an energy-dependent process (2, 51, 55). Thus, sensitivity to ATP depletion is one criterion for distinguishing active transport from diffusion. We therefore tested the effect of cytoplasmic ATP depletion on 5S rRNA nuclear import.

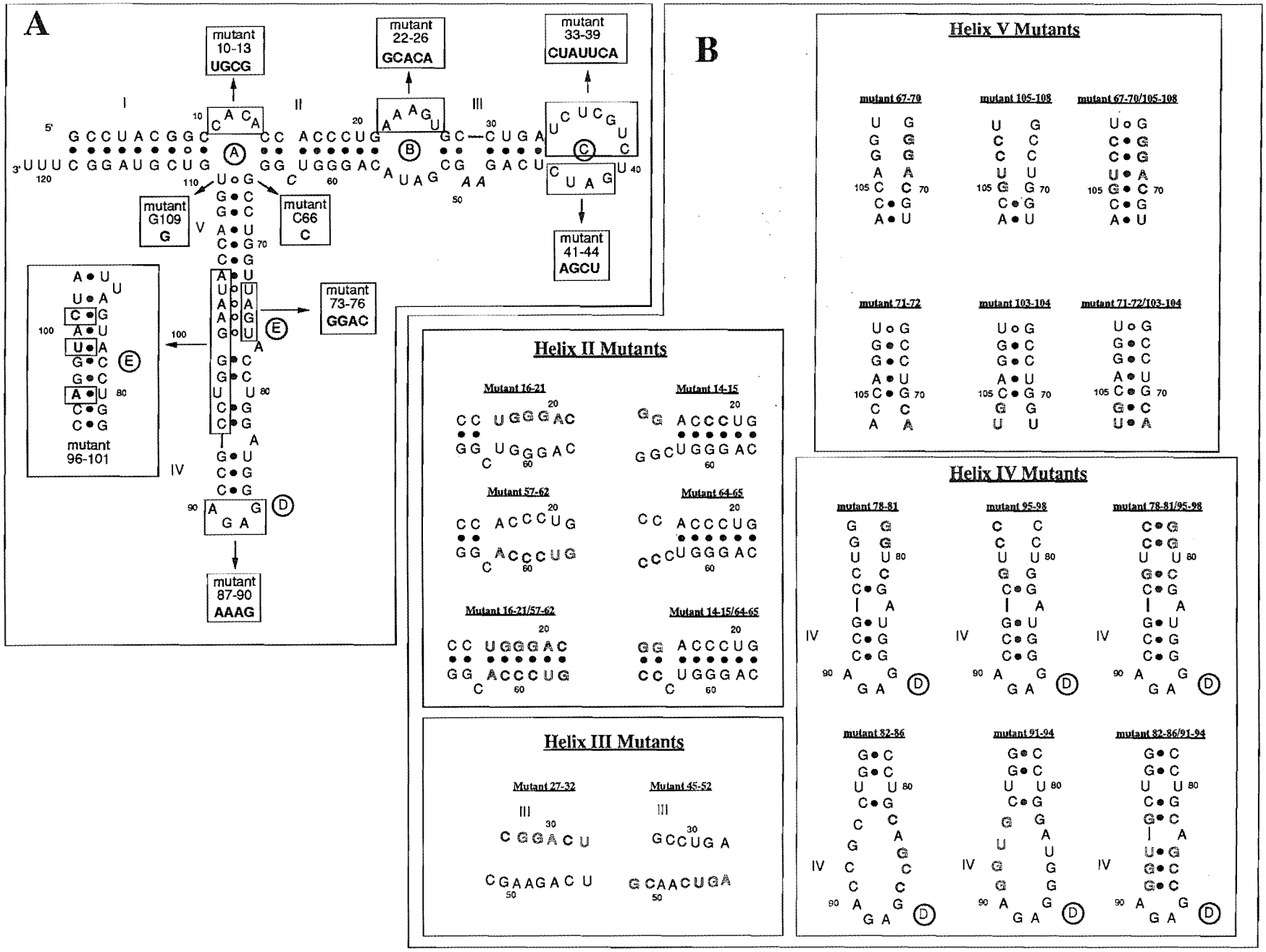


FIG. 1. Secondary structure of *X. laevis* oocyte-type 5S rRNA showing mutant nucleotide substitutions and deletions. (A) Single-strand substitution mutations. The bulged nucleotides deleted (A at nucleotides 49 and 50 and C at nucleotide 63) are indicated by italicized letters. (B) Helix mutants. Only the relevant region of the 5S rRNA is shown. Nucleotide substitutions are indicated by outlined letters.

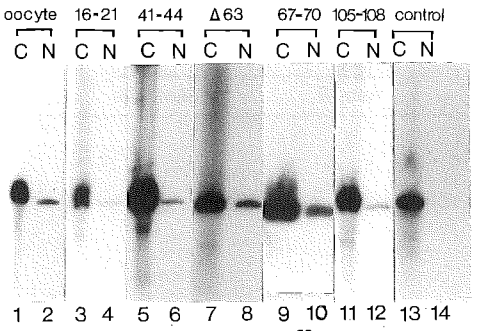


FIG. 2. Intracellular distribution of ^{32}P-labelled oocyte-type or mutant 5S rRNA species following microinjection in *X. laevis* oocytes. ^{32}P-labelled oocyte-type or mutant 5S rRNA was microinjected into the cytoplasm of fully grown oocytes. After overnight (18-h) incubation, RNA was extracted from five pooled nuclear (N) and cytoplasmic (C) fractions and separated electrophoretically on denaturing 8% polyacrylamide gels containing 8 M urea. The numbers refer to those nucleotides in the oocyte-type 5S rRNA (oocyte) which have been substituted or deleted (Fig. 1). A truncated 5S rRNA molecule consisting of nucleotides 1 to 98 (control) which does not enter the nucleus was included as a control for isolation of nuclei free of cytoplasmic contamination.

After injection of the ATP-hydrolyzing enzyme apyrase, ATP depletion was verified by a luciferase assay. At a final intracellular concentration of 100 U/ml, there was a rapid decline in ATP levels from 2 mM to 10 to 40 μM. This decrease occurred within 30 min and remained low after 25 h of incubation (data not shown). As shown in Fig. 3, ATP depletion significantly inhibited nuclear import of oocyte-type 5S rRNA (lane 4). The autoradiogram was deliberately overexposed to demonstrate the absence of detectable levels of labelled 5S rRNA in the nuclear fraction.

Another criterion for signal-mediated import via the nuclear pore complex is sensitivity to the lectin WGA. WGA is known to inhibit active transport by binding to *N*-acetylglucosamine (GlcNAc)-containing proteins present in the nuclear pore complex (25, 26, 65). Preinjection of WGA significantly inhibited nuclear import of oocyte-type 5S rRNA (Fig. 4A, lane 4). To verify that inhibition of import resulted from WGA interacting with GlcNAc-containing glycoproteins, WGA was coinjected with GlcNAc. Figure 4A (lane 6) shows that the presence of 50 mM GlcNAc abolished WGA-induced inhibition of import. The inhibitory effect of preinjected WGA on oocyte-type 5S rRNA was dose dependent (Fig. 4B), with 86% inhibition occurring at a concentration of 0.1 mg/ml. To ensure that WGA was not acting by physically occluding the nuclear pores, small fluorescently labelled dextrans (10 kDa) were microinjected into the oocyte cytoplasm. After overnight incubation, oocytes were dissected, and the nuclei were viewed by fluorescence microscopy. Preinjection of WGA had no effect on nonselective diffusion of small dextrans, and large fluorescently labelled dextrans (150 kDa) were excluded from the nucleus (data not shown). Similar results have been obtained in cultured somatic cells (15, 25).

An additional criterion for mediated transport is exclusion of nuclear proteins from the nucleus in chilled cells. Nuclear accumulation of large and small karyophilic proteins is inhibited by chilling, which slows enzyme-mediated reactions, whereas small proteins lacking nuclear localization signals may diffuse freely across the nuclear envelope, regardless of the temperature (10). Thus, 5S rRNA nuclear import was tested for temperature dependence. Figure 5 shows that the nuclear import of oocyte-type 5S rRNA was inhibited in chilled oocytes (lane 4). Chilling did not inhibit nonselective diffusion of a small fluorescent dextran into the nucleus (data not shown).

To determine whether nuclear import of mutant 5S rRNAs occurs by a mediated process or by nonselective diffusion, loop C mutant 41–44 and loop E mutant 96–101 were tested, since these RNAs have reduced abilities to enter the nucleus relative to that of oocyte-type 5S rRNA (Table 1). Preinjection of WGA significantly inhibited nuclear import of both mutant 5S rRNAs, although mutant 41–44 was less sensitive. Data are summarized in Table 2. Nuclear import of mutants 41–44 and 96–101 was also inhibited in chilled oocytes (Table 2), although both mutants were less sensitive than oocyte-type 5S rRNA. The possibility remains that nuclear entry of these mutants is partly diffusional; binding to an import receptor protein may be of low affinity or weakly temperature sensitive. Not all RNA-protein interactions, however, are temperature sensitive, because assembly of microinjected 5S rRNA into 7S RNPs (storage particles) does occur in chilled oocytes (data not shown).

In summary, nuclear import of 5S rRNA is sensitive to general inhibitors of nuclear pore-mediated translocation, so import is likely accomplished by a pathway similar to that used by karyophilic proteins.

5S rRNA is assembled into 60S ribosomal subunits in the nucleus. Although indirect evidence to date suggests that cytoplasmic 5S rRNA returns to the nucleus for ribosome incorporation (3, 18), a nuclear assembly site has not yet been demonstrated. To deduce the site of 5S rRNA assembly into 60S ribosomal subunits, labelled 5S rRNA was microinjected into the cytoplasm and subsequently immunoprecipitated from the nuclear versus cytoplasmic compartment.

The anti-60S ribosomal subunit serum detects two major bands in extracts of 60S ribosomal subunits (Fig. 6A and B, lanes 3); one band is approximately 37 kDa, and the other is approximately 14 to 15 kDa. The 37-kDa band corresponds to ribosomal protein L2; the 14- to 15-kDa band is not as easily identified but migrates similarly to ribosomal proteins in the range of L17 to L20 (53). This antiserum also reacts slightly with an approximately 18-kDa band in extracts of 40S ribosomal subunits (Fig. 6A and B, lanes 4). In the 7S fraction from a sucrose gradient, apart from the high-molecular-mass bands which probably represent polysaccharides, there is no cross-reaction (Fig. 6A and B, lanes 2). This antiserum does not immunoprecipitate 7S RNPs (Fig. 6C, lane 2). Intact ribosomes are immunoprecipitated by the anti-60S serum; the immunoprecipitates contain proteins that react with anti-40S ribosomal subunit serum as revealed by immunoblotting and contain both 28S and 18S rRNA as revealed by Northern blot (RNA) analysis (data not shown).

60S subunits containing labelled 5S rRNA were first faintly detectable in the nuclear fraction after 18 h but were not detectable in the cytoplasm (data not shown). After 48 h, labelled 5S rRNA was immunoprecipitated as 60S subunits from both the cytoplasmic fraction (Fig. 7, lane 2) and the nuclear fraction (lane 4), thus indicating a nuclear site of assembly, followed by export to the cytoplasm.

In a previous study, 7S RNPs were detected only in the oocyte cytoplasm after microinjection of labelled 5S rRNA and immunoprecipitation with anti-TFIIIA antibodies (3). Since TFIIIA has been shown to bind nuclear 5S rRNA (31), we repeated these assays with a different antibody preparation to determine whether a small nuclear pool of 7S RNPs was present. The antibodies raised against TFIIIA react with

TABLE 1. Summary of mutant phenotypes

Mutant designation[a]	Nuclear transport relative to oocytes type[b]	TFIIIA K_a in vitro[c]	Immunoprecipitation[d]		7S RNP EMSA[e]
			Anti-7S	Anti-60S	
Oocyte type	1.00	1.00	+	+	+
Helix II					
14–15 (HB)	0.49 ± 0.03 (3)	0.85 ± 0.22	IR	IR	+
64–65 (HB)	0.97 (1)	0.74 ± 0.24	−	+	+
14–15/64–65 (HM)	1.04 ± 0.16 (3)	1.11 ± 0.32	IR	+	+
16–21 (HB)	0.25 ± 0.05 (2)	0.32 ± 0.15	+	IR	ND
57–62 (HB)	0.73 (1)	0.40 ± 0.15	+	+	+
16–21/57–62 (HM)	1.88 (1)	1.09 ± 0.48	+	+	+
Helix III					
27–32 (HB)	0.70 ± 0.23 (2)	0.75 ± 0.10	+	+	+
45–52 (HB)	1.45 ± 0.34 (3)	0.76 ± 0.12	+	+	+
Helix IV					
78–81 (HB)	0.99 (1)	0.88 ± 0.01	+	+	ND
95–98 (HB)	0.86 (1)	0.78 ± 0.02	+	IR	+
78–81/95–98 (HM)	1.19 ± 0.46 (3)	0.86 ± 0.01	+	+	ND
82–86 (HB)	1.75 ± 0.02 (2)	0.81 ± 0.30	+	+	ND
91–94 (HB)	0.85 ± 0.31 (4)	0.96 ± 0.18	+	+	+
82–86/91–94 (HM)	1.38 ± 0.19 (2)	1.21 ± 0.35	+	+	ND
Helix V					
67–70 (HB)	1.18 ± 0.44 (2)	0.75 ± 0.12	−	+	+
105–108 (HB)	0.46 ± 0.15 (4)	0.39 ± 0.06	+	+	ND
67–70/105–108 (HM)	1.07 ± 0.34 (2)	0.71 ± 0.01	+	IR	+
71–72 (HB)	0.44 ± 0.12 (3)	0.35 ± 0.21	+	IR	ND
103–104 (HB)	0.48 ± 0.18 (3)	0.50 ± 0.23	+	IR	ND
71–72/103–104 (HM)	1.37 ± 0.43 (3)	1.18 ± 0.32	+	+	ND
Loop A					
10–13 (SEQ)	0.63 ± 0.00 (2)	0.30 ± 0.01	−	−	−
Loop B					
22–26 (SEQ)	1.18 (1)	1.00 ± 0.02	+	+	+
Loop C					
33–39 (SEQ)	0.97 ± 0.25 (3)	1.00 ± 0.02	+	+	+
41–44 (SEQ)	0.33 ± 0.06 (3)	0.40 ± 0.10	+	+	+
Loop D					
87–90 (SEQ)	1.26 (1)	0.71 ± 0.10	+	+	+
Loop E					
73–76 (SEQ)	1.60 ± 0.20 (2)	0.57 ± 0.02	−	+	+
96–101 (SEQ)	0.36 ± 0.04 (3)	0.59 ± 0.01	−	−	+
Bulged nucleotides					
Δ49,50 (DEL)	0.76 ± 0.38 (2)	1.00 ± 0.02	+	−	+
Δ63 (DEL)	1.04 ± 0.40 (4)	1.00 ± 0.02	+	−	+
Hinge nucleotides					
C66 (SEQ)	0.43 ± 0.05 (2)	0.12 ± 0.01	+	+	+
G109 (SEQ)	0.77 ± 0.02 (2)	0.17 ± 0.05	−	+	+

[a] The numbers in the designations refer to those nucleotides in 5S rRNA which have been substituted or deleted (Fig. 1). HB, helix breaking; HM, helix maintaining; SEQ, sequence mutation; DEL, deletion.

[b] Assays were performed as described in the legend to Fig. 2; nuclear transport was quantified by densitometry of suitable exposures of autoradiograms (within the linear range of signal intensity of the film), relative to the steady-state levels of oocyte-type 5S rRNA localized to the nucleus during each experiment. Experimental values are presented (mean ± standard deviation from the mean). Numbers in parentheses indicate the number of independent experiments performed.

[c] In vitro binding data is summarized from references 7, 8, 56, 58, and 74.

[d] Assays were performed as described in the legend to Fig. 8A. Protein binding was quantified by qualitative estimation of relative amounts of bound and free RNA. IR, inconclusive results caused by RNA with low specific activity.

[e] Electrophoretic mobility shift assays (EMSAs) were performed as described in the legend to Fig. 8C. ND, not determined.

the relevant protein (Fig. 6C, lane 1); no cross-reaction was noted with any other protein by immunoblotting (70). Under similar conditions, in this present study 7S RNPs containing labelled 5S rRNA were detectable only in the cytoplasmic fraction from 10 oocytes incubated for 21 h after microinjection (data not shown). However, a small nuclear pool of 7S RNPs was revealed by increasing the incubation time after microinjection to 48 h, increasing the specific activity of the labelled 5S rRNA, and increasing the sample size to 20 oocytes (Fig. 7, lane 3).

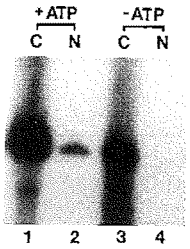


FIG. 3. Nuclear import of oocyte-type 5S rRNA is ATP dependent. Oocytes were preinjected with a final intracellular concentration of 100 U of apyrase per ml (−ATP), or PBS as a control (+ATP). After incubation for 30 min at 18°C, ^{32}P-labelled oocyte-type 5S rRNA was injected into the cytoplasm, and the oocytes were incubated for a further 6 h. The analysis for nuclear import was then performed as described in Fig. 2. C, cytoplasm; N, nucleus.

Sequence and structural requirements of 5S rRNA for 60S ribosomal subunit assembly and 7S RNP assembly in vivo. Since the results above demonstrated that the first requirement for subunit assembly is nuclear targeting, it was of interest to ascertain which sequence and structural elements of 5S rRNA are required for incorporation into 60S ribosomal subunits. Immunoprecipitation assays were thus performed on microinjected oocytes with antibodies against 60S subunits.

The results of several representative experiments are shown in Fig. 8A, and the complete data are presented in Table 1. Of 31 mutants, only 4 were completely defective in ribosome incorporation. Of these four mutants, two had mutations in single-strand loop regions, mutants 96–101 (Fig. 8A, lane 14) and 10–13 (lane 16), indicating that these regions of the molecule are important in recognition or binding of 5S rRNA with other ribosomal components. Comparable amounts of oocyte-type and mutant 5S rRNAs were detectable in the immunosupernatant fractions, demonstrating that the mutants were not degraded during the 48-h incubation (Fig. 8B). The simplest explanation for mutant 96–101 not being detected in ribosomes is that this results from impaired nuclear transport (Table 1). However, mutant 41–44 is equally defective in nuclear transport, yet it is found incorporated into 60S ribosomal subunits (lane 12). The other two defective mutants were Δ49,50 (lane 18) and Δ63 (lane 20), indicating that these bulged nucleotides are important for 5S rRNA incorporation into the 60S subunit. Results were inconclusive for six mutants (Table 1). The possibility that the absence of these labelled RNAs in 60S ribosomal subunits was due to the low specific activity of the RNA could not be ruled out. Despite repeated attempts to increase the specific activity, these particular mutant 5S rRNA gene constructs continued to provide poor templates for transcription.

In addition to assaying different mutant 5S rRNA molecules for their incorporation into 60S subunits, immunoprecipitation of 7S RNPs was also performed with anti-TFIIIA antibodies. This qualitative analysis provided a comparison with the in vitro data previously obtained for TFIIIA binding to the mutant 5S rRNA molecules (summarized in Table 1). All but six mutant 5S rRNAs were found to be able to bind TFIIIA in the oocyte in an immunodetectable complex. One of these defective mutants, 10–13 (Fig. 8A, lane 15), corresponds with the in vitro data; mutant 10–13 has a K_a of 0.30 and is 20 times less effective in inhibiting TFIIIA binding than oocyte-type 5S rRNA is (56). Negative results with other mutants, however, were contradictory. For example,

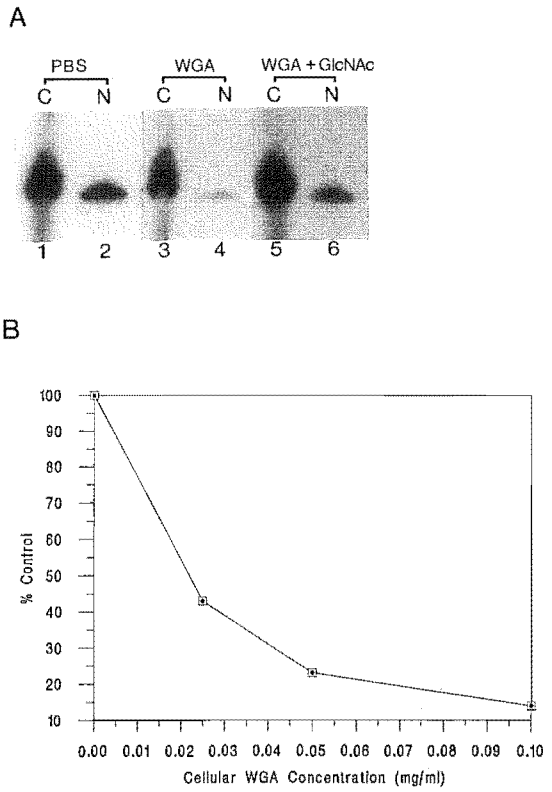


FIG. 4. Nuclear import of oocyte-type 5S rRNA is inhibited by WGA. (A) Oocytes were preinjected with WGA at a final intracellular concentration of 0.1 mg/ml or with WGA (0.1 mg/ml) and 50 mM *N*-acetylglucosamine (WGA + GlcNAc). PBS was preinjected as a control. After incubation for 3 h, ^{32}P-labelled oocyte-type 5S rRNA was injected into the cytoplasm and the oocytes were incubated for a further 18 h. The analysis for nuclear import was then performed as described in the legend to Fig. 2. C, cytoplasm; N, nucleus. (B) Dose-dependent inhibition of 5S rRNA nuclear import. Oocytes were preinjected with 50 nl of WGA at various concentrations. The final intracellular concentration of WGA is denoted on the x axis. The nuclear import of ^{32}P-labelled 5S rRNA was assayed 18 h after injection. Import in the presence of WGA is expressed as a percentage of the import of 5S rRNA in control oocytes preinjected with PBS only (% Control).

mutant 67–70 (Fig. 8A, lane 7) was not immunoprecipitated with anti-TFIIIA yet has an in vitro binding affinity of 0.75 (74). To clarify these results, homogenates of microinjected oocytes were analyzed by electrophoretic mobility shift assays. As shown in Fig. 8C, mutant 67–70 (lane 5) clearly showed a band shift present at a position corresponding to that of 7S RNPs. Similarly, mutants 64–65, 73–76, 96–101, and G109 which were not immunodetectable by anti-TFIIIA showed band shifts corresponding to 7S RNPs (Fig. 8A and C and Table 1). In contrast, consistent with the in vitro binding data and immunoprecipitation data, no band shift corresponding to 7S RNPs was detected with mutant 10–13 (Fig. 8C, lane 8). The data are summarized in Table 1.

The inconsistent anti-TFIIIA immunoprecipitation assay and electrophoretic mobility shift assay results described above can be explained by reasoning that for these mutant 5S rRNA molecules, the 5S rRNA-TFIIIA complexes that are formed have different conformations which are not recognized by the anti-TFIIIA antibody or that the complexes formed are not stable under the assay conditions.

FIG. 5. Effect of chilling on the nuclear import of oocyte-type 5S rRNA. ^{32}P-labelled oocyte-type 5S rRNA was injected into the oocyte cytoplasm. After incubation for 18 h at either 18°C as a control or on ice (0°C), the analysis for nuclear import was performed as described in the legend to Fig. 2. C, cytoplasm; N, nucleus.

These observations raise a cautionary note toward relying solely on antigen-antibody interactions for the analysis of RNA-protein interactions.

The potential formation of an RNA-protein complex not recognized by an antibody is not a likely consideration with the anti-60S ribosomal subunit antibody. 5S rRNA is thought to be buried in the 60S subunit (42), within the peptidyl transferase center, whereas the ribosomal proteins recognized by this antibody (L2 and another in the range of L17 to L20) are not detected in this region (23, 63, 68). Given the apparent lack of direct cross-links between 5S rRNA and the ribosomal proteins recognized by the anti-60S antibody, it is unlikely that any conformational change resulting from incorporation of a mutant 5S rRNA molecule into the 60S subunit would be transmitted to these ribosomal proteins; thus, the recognition or binding of the anti-60S antibody to these epitopes would not be affected.

TABLE 2. Summary of effects of WGA and chilling on nuclear import of oocyte-type and mutant 5S rRNA

RNA	% Control[a]	
	WGA	0°C
Oocyte type	14.6 (±0.4)	4.1 (±2.1)
41–44	42.0 (±3.4)	43.0 (±3.0)
96–101	17.0 (±1.6)	22.0

[a] Nuclear import of the designated ^{32}P-labelled RNA (Fig. 1) was assayed in samples of five oocytes as described in the legend to Fig. 4A for WGA (final intracellular concentration of 0.2 mg/ml) and in the legend to Fig. 5 for chilling. Import was quantified by densitometry and expressed as a percentage (± standard deviation from the mean) of the amount of import in control oocytes (% Control). Tabulated results are from two samples, except for mutant 96–101 (0°C) for which one sample was used.

In summary, specific structural elements of the 5S rRNA molecule have been shown to be critical for ribosome incorporation. Deletion of bulged nucleotides and nucleotide substitutions that alter the sequence of loop A or the noncanonical base pairing in loop E (57, 72) abolish ribosome incorporation. Some of the structural elements of 5S rRNA required for ribosome incorporation differ from those elements required for nuclear transport. Further, some of the structural requirements of 5S rRNA for 7S RNP assembly and for 60S ribosomal subunit assembly differ.

DISCUSSION

From the 5S rRNA mutants studied in this report, a picture emerges of complex and nonidentical structural features within the central domain of the RNA molecule that

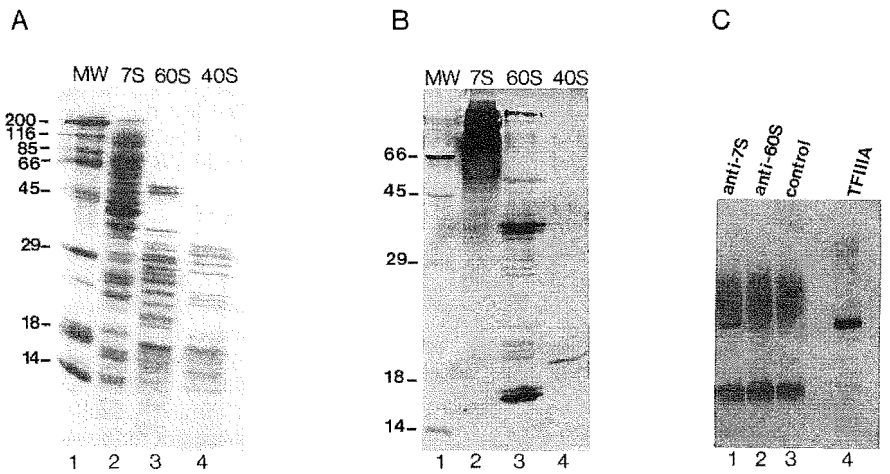


FIG. 6. Control of antiserum specificity by immunoblotting and immunoprecipitation assays. Total protein from 7S fractions and from ribosomal subunits purified from *Xenopus* ovaries was fractionated by electrophoresis in 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) and either stained with Coomassie blue (A) or transferred to Immobilon-P polyvinylidene difluoride membranes and probed with anti-60S ribosomal subunit antiserum (B). The antigen-antibody complexes were revealed by treatment with ^{35}S-labelled protein A and then autoradiography (48 h). The high-molecular-mass bands in lane 2 of panel B are probably polysaccharides. Lanes: MW, protein molecular mass markers (in kilodaltons); 7S, approximately 10 μg of protein purified from the 7S region of a sucrose gradient; 60S, approximately 3 μg of protein from 60S ribosomal subunits purified by sucrose gradient centrifugation; 40S, approximately 1 μg of protein from 40S ribosomal subunits purified by sucrose gradient centrifugation. (C) 7S RNPs purified from immature *Xenopus* ovaries were incubated with protein A-Sepharose-antibody complexes in an immunoprecipitation assay. Proteins were recovered and separated electrophoretically on 12% polyacrylamide gels containing 0.1% SDS and then silver stained. Lanes: anti-7S, immunoprecipitation with anti-TFIIIA; anti-60S, immunoprecipitation with anti-60S ribosomal subunit; control, mock immunoprecipitation with anti-60S antiserum, but without 7S RNPs; TFIIIA, TFIIIA protein from 7S RNPs.

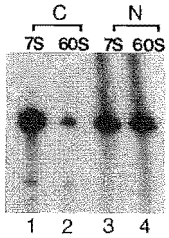


FIG. 7. Intracellular localization of microinjected 5S rRNA in 60S ribosomal subunits and 7S RNPs. Labelled 5S rRNA was microinjected into the cytoplasm of oocytes. After incubation for 48 h, 20 oocytes were manually fractionated into cytoplasmic (C) and nuclear (N) compartments. Homogenates from each compartment were incubated with protein A-Sepharose-antibody complexes in an immunoprecipitation assay. Labelled RNAs were recovered and separated electrophoretically on 8% polyacrylamide gels containing 8 M urea. Lanes: 7S, immunoprecipitation with anti-TFIIIA; 60S, immunoprecipitation with anti-60S ribosomal subunit.

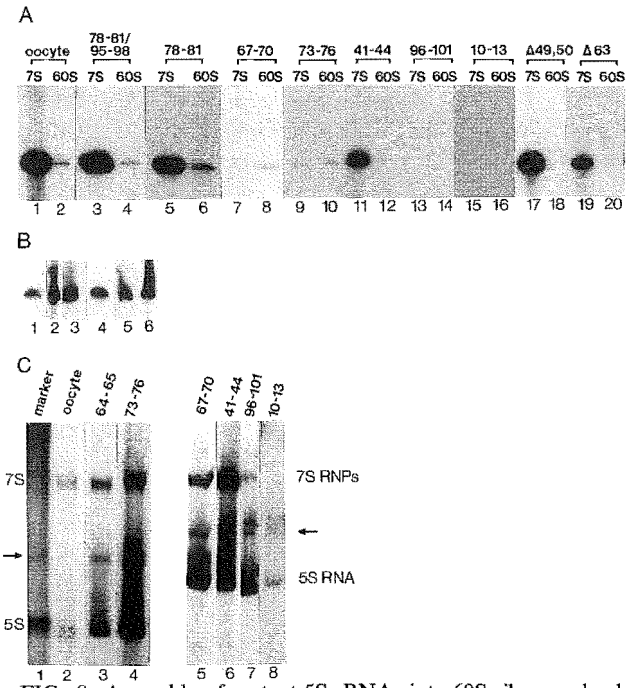


FIG. 8. Assembly of mutant 5S rRNAs into 60S ribosomal subunits and 7S RNPs. (A) ^{32}P-labelled oocyte-type or mutant 5S rRNA was microinjected into the oocyte cytoplasm. After 48 h, homogenates of whole oocytes were analyzed by immunoprecipitation assay as described in the legend to Fig. 7. Labelled RNAs were recovered and separated electrophoretically on 8% polyacrylamide gels containing 8 M urea. The numbers in the mutant designations refer to those nucleotides in oocyte-type 5S rRNA (oocyte) which have been substituted or deleted (Fig. 1). Lanes: 7S, immunoprecipitation with anti-TFIIIA; 60S, immunoprecipitation with anti-60S ribosomal subunit. (B) Labelled RNAs recovered from immunosupernatant fractions, from the anti-60S immunoprecipitation assays in panel A. Lane 1, oocyte-type RNA; lane 2, mutant 67–70; lane 3, mutant 96–101; lane 4, mutant 10–13; lane 5, mutant Δ49,50; lane 6, mutant Δ63. (C) Electrophoretic gel mobility shift assays of mutant 5S rRNAs. Labelled oocyte-type or mutant 5S rRNA was injected into the oocyte cytoplasm. After 48 h, homogenates were electrophoresed on nondenaturing 6% polyacrylamide gels containing 0.1% Triton X-100. The location of 7S RNPs was determined by running an additional lane of unlabelled, purified 7S RNPs; this lane was stained with ethidium bromide and visualized by UV illumination (not shown). The arrows indicate a band shift that may represent the 5S rRNA-ribosomal protein L5 complex, but this has yet to be confirmed. Marker, ^{32}P-labelled 5S rRNA. Lanes 1 to 4 and lanes 5 to 8 represent experiments in which samples were electrophoresed for different lengths of time.

are required for nuclear transport, assembly into 7S RNPs (storage particles) and assembly into 60S ribosomal subunits (Fig. 9). 5S rRNA is assembled into 60S ribosomal subunits in the nucleus; thus, the first requirement for subunit assembly is nuclear targeting. However, some of the structural elements of 5S rRNA required for ribosome incorporation differ from those elements required for nuclear transport (Fig. 9). Differences in the requirements for nuclear transport and TFIIIA binding suggest that TFIIIA binding is not a prerequisite for nuclear targeting of 5S rRNA.

Subcellular trafficking of 5S rRNA. Since 5S rRNA can be easily and specifically dissociated from intact ribosomes or subunits as a 5S rRNA-L5 complex (3), it was thought that the 5S rRNA might be added as a surface component to partially assembled subunits, hence a cytoplasmic site of integration of stored 5S rRNA seemed plausible. However, results presented here indicate that cytoplasmically stored 5S rRNA returns to the nucleus for assembly into the large 60S subunit. This conclusion is consistent with a recent study in *Saccharomyces cerevisiae*, which suggests that 5S rRNA is crucial to an early step in subunit assembly (69). Thus, analysis of intracellular distribution reflects the steady-state levels of 5S rRNA molecules reached after overnight incubation (3); 5S rRNA is released from storage, migrates into the nucleus, is assembled into ribosomes, and then is exported to the cytoplasm. Since ribosome assembly continues for a protracted period of time in *Xenopus* oocytes, only a small fraction of microinjected 5S rRNA would be expected to appear in the nucleus at any given time or be incorporated into nascent ribosomes. The latter is reflected in the small fraction of labelled 5S rRNA found in 60S subunits compared with 7S RNPs (storage particles). Interestingly, a number of mutants accumulated in the nucleus to a greater degree than oocyte-type 5S rRNA, for example, nuclear accumulation of mutant 16–21/57–62 was nearly twice that of oocyte-type 5S rRNA. It is not clear whether this distribution represents enhanced nuclear import or whether the increased nuclear accumulation of some mutants is due to a defect in another function, such as nuclear export.

Mediated nuclear import of 5S rRNA. Nuclear import of 5S rRNA is inhibited by cytoplasmic ATP depletion, WGA, and chilling. Similarly, nuclear protein import (2, 51, 55), export of ribosomal subunits (6), and mRNA export (16) require metabolic energy in vivo. The concentration of WGA required to inhibit 5S rRNA nuclear import is comparable to the concentration of WGA shown to inhibit the nuclear import of U6 small nuclear RNA, but 20 times less than the concentration required to inhibit nuclear import of U1 small nuclear RNA to a similar degree (see Fig. 3 in reference 48). These results correlate with studies on nuclear export of 5S rRNA and 5S rRNA-containing RNPs. Approximately 60% inhibition of ribosomal subunit export can be achieved when WGA is injected into the nucleus at a final concentration of 0.5 mg/ml (6), and export of 5S rRNA newly synthesized from microinjected cloned genes is significantly inhibited by preinjection of RL1, an antinucleoporin monoclonal antibody (24). The sensitivity of 5S rRNA to general inhibitors of nuclear transport suggests that 5S rRNA is targeted to oocyte nuclei by a receptor-mediated process.

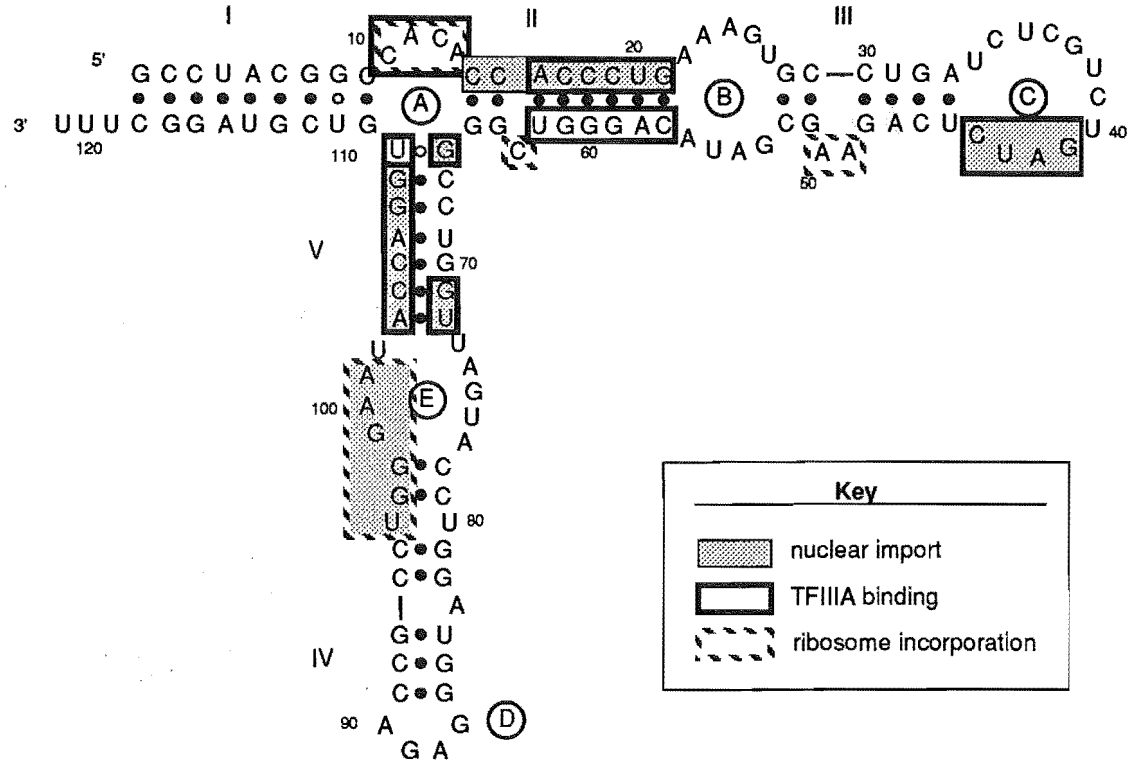


FIG. 9. Complex, nonidentical regions of 5S rRNA required for different functions. Shaded boxes indicate regions in which nucleotide substitution results in at least a 50% reduction in nuclear transport of 5S rRNA. Boxes outlined in bold indicate regions in which nucleotide substitution results in at least a 50% reduction in TFIIIA binding affinity. Boxes outlined in dashed lines indicate regions in which nucleotide substitution abolishes incorporation of 5S rRNA into the 60S ribosomal subunit.

Given the specificity of receptor-mediated processes, it seemed surprising that all mutants were capable of at least some degree of nuclear import. However, the results presented here are similar to an analysis of the nuclear export phenotypes of 30 different point mutants of human $tRNA_i^{Met}$ which revealed variable defects in transport; the percentage of microinjected tRNA exported to the cytoplasm ranged from 85 (wild type) to 34% (D stem mutant) (67). Glyceraldehyde-3-phosphate dehydrogenase binds two defective mutants with lower affinity than that of wild-type tRNA, suggesting that this protein may be involved in tRNA export (62). This comparison with tRNA export and a model for the tertiary structure of 5S rRNA provide some insight into 5S rRNA import. 5S rRNA has been proposed to adopt a Y-shaped structure in which the three helical domains are independent (13, 71), that is, the effect that a mutation has on the RNA structure is primarily restricted to the mutated loop (12, 40). If a protein involved in nuclear targeting makes numerous contacts with 5S rRNA, disruption of one small region may not disrupt binding in other regions, therefore potentially providing a stable complex that is still recognized by the transport machinery. Relatively small changes in complex stability may be masked at the saturating levels of RNA used in these experiments.

Guddat et al. (31) proposed that 5S rRNA nuclear export is mediated by either TFIIIA or L5 on the basis of studies showing that mutant RNA molecules that do not form immunodetectable complexes with these proteins are retained in the nucleus. Results of this present study suggest that TFIIIA binding is not a prerequisite for nuclear import of 5S rRNA, consistent with earlier work suggesting that 7S RNP transport is unidirectional (3, 44). A small nuclear pool of 7S RNPs containing labelled 5S rRNA was detected after microinjection of labelled 5S rRNA into the oocyte cytoplasm, but it is likely that these 7S RNPs were assembled after nuclear entry. If 5S rRNA enters the nucleus in excess of the amount required for assembly into nascent 60S ribosomal subunits, it may become associated with TFIIIA. Along these lines, it has been proposed that excess 5S rRNA may be targeted to the cytoplasm of mammalian somatic cells for degradation bound to a TFIIIA-like protein (39).

Ribosomal protein L5 and 5S rRNA form a stable complex prior to assembly of ribosomal subunits (3, 11, 64, 73). Cytoplasmic exchange between TFIIIA and L5 for binding of 5S rRNA correlates with the mobilization of 5S rRNA from storage within the *Xenopus* oocyte cytoplasm (3), suggesting that L5 may mediate 5S rRNA nuclear targeting. Little is known about the sequence and structural requirements for L5 binding to 5S rRNA, although chemical protection assays (35) and immunoprecipitation assays (3) indicate a binding domain similar to that of TFIIIA. Delineation of the sequence and structural requirements of 5S rRNA for L5 binding and correlation with data on subcellular localization should provide further insight into the role of L5 in nuclear import and nucleolar targeting of 5S rRNA. The possibility remains that other proteins, such as carriers that shuttle between the cytoplasm and the nucleus (9, 36, 46, 60), are involved in the subcellular trafficking of 5S rRNA.

5S rRNA structural elements required for ribosome assembly. Loop structures, bulged nucleotides, and non-Watson-

Crick base pairs of RNA have been shown to be of importance for specific protein recognition (4, 14, 54). Similarly, results presented here show that incorporation of 5S rRNA into ribosomes is abolished by deletion of bulged nucleotides, A at nucleotides 49 and 50 or C at nucleotide 63, and by nucleotide substitutions that alter the sequence of loop A or the noncanonical base pairing in loop E. These results correlate with results from other workers investigating prokaryotic 5S rRNA-protein interactions. *Escherichia coli* 5S rRNA has a structure similar to that of eukaryotic 5S rRNA, but *E. coli* 5S rRNA binds to three ribosomal proteins, L5, L18, and L25, rather than to one protein as in eukaryotes. A bulged nucleotide at position 65 in *E. coli* 5S rRNA, corresponding to position 63 in eukaryotic 5S rRNA, is necessary for binding of L18 to the 5S rRNA (reviewed in reference 29).

Various regions of 5S rRNA have been postulated to be of importance for ribosome assembly and function (69; for a review, see reference 29). Complementary base pairing between nucleotide sequences at the 5′ and 3′ ends of mouse 18S rRNA contained in the 40S ribosomal subunit and nucleotides 9 to 26 and 90 to 107 of 5S rRNA occurs in vitro (59). The functional significance of this stable interaction has yet to be determined. Interestingly, mutant 96–101 which has increased Watson-Crick base pairing in the loop E-helix IV region of 5S rRNA and mutant 10–13 which has an altered sequence in loop A were shown here to be defective in ribosome incorporation. These results indicate a correlation between the structural elements of the 5S rRNA molecule required for 60S ribosomal subunit assembly and the structural elements previously proposed to aid in formation of the 80S ribosome by 5S rRNA-nucleic acid interactions. Protein recognition of the noncanonical base pairing in loop E encompassing nucleotides 96 to 101 of the 5S rRNA molecule (57, 72) may be of importance for 60S ribosomal subunit assembly, as well as RNA-RNA hybridization.

5S rRNA represents an important model system for study of the regulated subcellular trafficking of RNA, because it involves many different components: shuttling of the 5S rRNA molecule across the nuclear envelope, a variety of RNA-protein interactions and exchanges, cytoplasmic localization of the 5S rRNA in RNPs (storage particles), and nucleolar targeting. Continued study of the effects of mutations by an in vitro binding assay (7, 8, 56, 74) and by the in vivo functional assays described here should help to further identify and clarify those sequence and structural elements of 5S rRNA required for some of its biological activities.

ACKNOWLEDGMENTS

This research was supported in part by grants from the University Grants Committee, Lottery Grants Board, and Health Research Council of New Zealand (to L.A.A.) and from the Natural Sciences and Engineering Research Council of Canada (NSERC) (to P.J.R.). P.J.R. is the recipient of an NSERC University Research Fellowship.

We are grateful to Renny Bishop for maintaining the *Xenopus* colony. We thank Alain Viel (Harvard University) and Adrienne Gomez de Gracia (Centre de Génétique Moléculaire) for help in preparation of the antisera used in this study. Thanks also are due to Dave Goldfarb and Neil Michaud (University of Rochester) for critical discussions of preliminary results and to Aimée Bakken (University of Washington) for helpful discussions.

REFERENCES

1. **Adam, S. A., and L. Gerace.** 1991. Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. Cell **66**:837–847.
2. **Akey, C. W., and D. S. Goldfarb.** 1989. Protein import through the nuclear pore complex is a multistep process. J. Cell Biol. **109**:971–982.
3. **Allison, L. A., P. J. Romaniuk, and A. H. Bakken.** 1991. RNA-protein interactions of stored 5S RNA with TFIIIA and ribosomal protein L5 during *Xenopus* oogenesis. Dev. Biol. **144**:129–144.
4. **Bartel, D. P., M. L. Zapp, M. R. Green, and J. W. Szostak.** 1991. HIV-1 Rev regulation involves recognition of non-Watson-Crick base pairs in viral RNA. Cell **67**:529–536.
5. **Baserga, S. J., M. Gilmore-Hebert, and X. W. Yang.** 1992. Distinct molecular signals for nuclear import of the nucleolar snRNA, U3. Genes Dev. **6**:1120–1130.
6. **Bataillé, N., T. Helser, and H. M. Fried.** 1990. Cytoplasmic transport of ribosomal subunits microinjected into the *Xenopus laevis* oocyte nucleus: a generalized, facilitated process. J. Cell Biol. **111**:1571–1582.
7. **Baudin, F., and P. J. Romaniuk.** 1989. A difference in the importance of bulged nucleotides and their parent base pairs in the binding of transcription factor IIIA to *Xenopus* 5S RNA and 5S RNA genes. Nucleic Acids Res. **17**:2043–2056.
8. **Baudin, F., P. J. Romaniuk, P. Romby, C. Brunel, E. Westhof, B. Ehresmann, and C. Ehresmann.** 1991. Involvement of "hinge" nucleotides of *Xenopus laevis* 5 S rRNA in the RNA structural organization and in the binding of transcription factor TFIIIA. J. Mol. Biol. **218**:69–81.
9. **Borer, R. A., C. F. Lehner, H. M. Eppenberger, and E. A. Nigg.** 1989. Major nucleolar proteins shuttle between nucleus and cytoplasm. Cell **56**:379–390.
10. **Breeuwer, M., and D. S. Goldfarb.** 1990. Facilitated nuclear transport of histone H1 and other small nucleophilic proteins. Cell **60**:999–1008.
11. **Brow, D. A., and E. P. Geiduschek.** 1987. Modulation of yeast 5S RNA synthesis in vitro by ribosomal protein YL3: a possible regulatory loop. J. Biol. Chem. **2621**:13953–13958.
12. **Brunel, C., P. Romby, E. Westhof, P. J. Romaniuk, B. Ehresmann, and C. Ehresmann.** 1990. Effect of mutations in domain 2 on the structural organization of oocyte 5 S rRNA from *Xenopus laevis*. J. Mol. Biol. **215**:103–111.
13. **Chow, C. S., K. M. Hartmann, S. L. Rawlings, P. W. Huber, and J. K. Barton.** 1992. Delineation of structural domains in eukaryotic 5S rRNA with a rhodium probe. Biochemistry **31**:3534–3542.
14. **Cullen, B. R.** 1990. The HIV-1 Tat protein: an RNA sequence-specific processivity factor? Cell **63**:655–657.
15. **Dabauvalle, M.-C., B. Schulz, U. Scheer, and R. Peters.** 1988. Inhibition of nuclear accumulation of karyophilic proteins in living cells by microinjection of the lectin wheat germ agglutinin. Exp. Cell Res. **174**:291–296.
16. **Dargemont, C., and L. C. Kühn.** 1992. Export of mRNA from microinjected nuclei of *Xenopus laevis* oocytes. J. Cell Biol. **118**:1–9.
17. **Denis, H., and M. le Maire.** 1983. Thesaurisomes, a novel kind of nucleoprotein particle, p. 263–267. *In* D. B. Roodyn (ed.), Subcellular biochemistry. Plenum Publishing Corp., New York.
18. **De Robertis, E. M., S. Lienhard, and R. F. Parisot.** 1982. Intracellular transport of microinjected 5S and small nuclear RNAs. Nature (London) **295**:572–577.
19. **Draper, D. E.** 1989. How do proteins recognize specific RNA sites? New clues from autogenously regulated ribosomal proteins. Trends Biochem. Sci. **14**:335–338.
20. **Dworetzky, S. I., and C. M. Feldherr.** 1988. Translocation of RNA-coated gold particles through the nuclear pores of oocytes. J. Cell Biol. **106**:575–584.
21. **Dworetzky, S. I., R. E. Lanford, and C. M. Feldherr.** 1988. The effects of variations in the number and sequence of targeting signals on nuclear uptake. J. Cell Biol. **107**:1279–1287.
22. **Eckner, R., W. Ellmeier, and M. L. Birnstiel.** 1991. Mature mRNA 3′ end formation stimulates RNA export from the nucleus. EMBO J. **10**:3513–3522.
23. **Fabijanski, S., and M. Pellegrini.** 1981. Identification of proteins at the peptidyl-tRNA binding site of rat liver ribosomes. Mol. Gen. Genet. **184**:551–556.

24. **Featherstone, C., M. K. Darby, and L. Gerace.** 1988. A monoclonal antibody against the nuclear pore complex inhibits nucleocytoplasmic transport of protein and RNA *in vivo*. J. Cell Biol. **107**:1289–1297.
25. **Finlay, D. R., and D. J. Forbes.** 1990. Reconstitution of biochemically altered nuclear pores: transport can be eliminated and restored. Cell **60**:17–29.
26. **Finlay, D. R., E. Meier, P. Bradley, J. Horecka, and D. J. Forbes.** 1991. A complex of nuclear pore proteins required for pore function. J. Cell Biol. **114**:169–183.
27. **Fischer, U., E. Darzynkiewicz, S. M. Tahara, N. A. Dathan, R. Lührmann, and I. W. Mattaj.** 1991. Diversity in the signals required for nuclear accumulation of U snRNPs and variety in the pathways of nuclear transport. J. Cell Biol. **113**:705–714.
28. **Fischer, U., V. Sumpter, M. Sekine, T. Satoh, and R. Lührmann.** 1993. Nucleocytoplasmic transport of U snRNPs: definition of a nuclear location signal in the Sm core domain that binds a transport receptor independently of the m_3G cap. EMBO J. **12**:573–583.
29. **Garrett, R. A., S. Douthwaite, and H. F. Noller.** 1981. Structure and role of 5S RNA-protein complexes in protein biosynthesis. Trends Biochem. Sci. **6**:137–139.
30. **Goldfarb, D., and N. Michaud.** 1991. Pathways for the nuclear transport of proteins and RNAs. Trends Cell Biol. **1**:20–24.
31. **Guddat, U., A. H. Bakken, and T. Pieler.** 1990. Protein-mediated nuclear export of RNA: 5S rRNA containing small RNPs in Xenopus oocytes. Cell **60**:619–628.
32. **Hamm, J., E. Darzynkiewicz, S. M. Tahara, and I. W. Mattaj.** 1990. The trimethylguanosine cap structure of U1 snRNA is a component of a bipartite nuclear targeting signal. Cell **62**:569–577.
33. **Hamm, J., and I. W. Mattaj.** 1990. Monomethylated cap structures facilitate RNA export from the nucleus. Cell **63**:109–118.
34. **Holmberg, L., Y. Melander, and O. Nygård.** 1992. Ribosome-bound eukaryotic elongation factor 2 protects 5S rRNA from modification. J. Biol. Chem. **267**:21906–21910.
35. **Huber, P. W., and I. G. Wool.** 1986. Use of the cytotoxic nuclease α-sarcin to identify the binding site on eukaryotic 5 S ribosomal ribonucleic acid for the ribosomal protein L5. J. Biol. Chem. **261**:3002–3005.
36. **Imamoto, N., Y. Matsuoka, T. Kurihara, K. Kohno, M. Miyagi, F. Sakiyama, Y. Okada, S. Tsunasawa, and Y. Yoneda.** 1992. Antibodies against 70-kD heat shock cognate protein inhibit mediated nuclear import of karyophilic proteins. J. Cell Biol. **119**:1047–1061.
37. **Kelly, J. M., and R. A. Cox.** 1982. The nucleotide sequence at the 3′-end of *Neurospora crassa* 18S-rRNA and studies on the interaction with 5S-rRNA. Nucleic Acids Res. **10**:6733–6745.
38. **Khanna-Gupta, A., and V. C. Ware.** 1989. Nucleocytoplasmic transport of ribosomes in a eukaryotic system: is there a facilitated transport process? Proc. Natl. Acad. Sci. USA **86**:1791–1795.
39. **Lagaye, S., J.-P. Barque, M. le Maire, H. Denis, and C.-J. Larsen.** 1988. Characterization by human antibodies of two HeLa cell proteins which are related to *Xenopus laevis* transcription factor TFIIIA. Nucleic Acids Res. **16**:2473–2487.
40. **Leal de Stevenson, I., P. Romby, F. Baudin, C. Brunel, E. Westhof, C. Ehresmann, B. Ehresmann, and P. J. Romaniuk.** 1991. Structural studies on site-directed mutants of domain 3 of *Xenopus laevis* oocyte 5 S ribosomal RNA. J. Mol. Biol. **219**:243–255.
41. **le Maire, M., and H. Denis.** 1987. Biochemical research on oogenesis. Binding of tRNA to the nucleoprotein particles of *Xenopus laevis* previtellogenic oocytes. J. Biol. Chem. **262**:654–659.
42. **Lo, A. C., and R. N. Nazar.** 1982. Topography of 5.8 S rRNA in rat liver ribosomes. J. Biol. Chem. **257**:3516–3524.
43. **Martin, K., and A. Helenius.** 1991. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. Cell **67**:117–130.
44. **Mattaj, I. W., S. Lienhard, R. Zeller, and E. M. DeRobertis.** 1983. Nuclear exclusion of transcription factor IIIA and the 42S particle transfer RNA-binding protein in *Xenopus* oocytes: a possible mechanism for gene control? J. Cell Biol. **97**:1261–1265.
45. **Mehlin, H., B. Daneholt, and U. Skoglund.** 1992. Translocation of a specific pre-messenger ribonucleoprotein particle through the nuclear pore studied with electron microscope tomography. Cell **69**:605–613.
46. **Meier, U. T., and G. Blobel.** 1992. Nopp140 shuttles on tracks between nucleolus and cytoplasm. Cell **70**:127–138.
47. **Michaud, N., and D. S. Goldfarb.** 1991. Multiple pathways in nuclear transport: the import of U2 snRNP occurs by a novel kinetic pathway. J. Cell Biol. **112**:215–224.
48. **Michaud, N., and D. Goldfarb.** 1992. Microinjected U snRNAs are imported to oocyte nuclei via the nuclear pore complex by three distinguishable targeting pathways. J. Cell Biol. **116**:851–861.
49. **Moore, M. S., and G. Blobel.** 1992. The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. Cell **69**:939–950.
50. **Neuman de Vegvar, H. E., and J. D. Dahlberg.** 1990. Nucleocytoplasmic transport and processing of small nuclear RNA precursors. Mol. Cell. Biol. **10**:3365–3375.
51. **Newmeyer, D. D., and D. J. Forbes.** 1988. Nuclear import can be separated into distinct steps in vitro: nuclear pore binding and translocation. Cell **52**:641–653.
52. **Peters, R.** 1984. Nucleo-cytoplasmic flux and intracellular mobility in single hepatocytes measured by fluorescence microphotolysis. EMBO J. **3**:1831–1836.
53. **Pierandrei-Amaldi, P., and E. Beccari.** 1980. Messenger RNA for ribosomal proteins in *Xenopus laevis* oocytes. Eur. J. Biochem. **106**:603–611.
54. **Pruijn, G. J. M., R. L. Slobbe, and W. J. van Venrooij.** 1991. Analysis of protein-RNA interactions within Ro ribonucleoprotein complexes. Nucleic Acids Res. **19**:5173–5180.
55. **Richardson, W. D., A. D. Mills, S. M. Dilworth, R. A. Laskey, and C. Dingwall.** 1988. Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. Cell **52**:655–664.
56. **Romaniuk, P. J.** 1989. The role of highly conserved single-stranded nucleotides of *Xenopus* 5S RNA in the binding of transcription factor IIIA. Biochemistry **28**:1388–1395.
57. **Romaniuk, P. J., I. Leal de Stevenson, C. Ehresmann, P. Romby, and B. Ehresmann.** 1988. A comparison of the solution structures and conformational properties of the somatic and oocyte 5S rRNAs of *Xenopus laevis*. Nucleic Acids Res. **16**:2295–2312.
58. **Romaniuk, P. J., I. Leal de Stevenson, and H.-H. A. Wong.** 1987. Defining the binding site of *Xenopus* transcription factor IIIA on 5S RNA using truncated and chimeric 5S RNA molecules. Nucleic Acids Res. **15**:2737–2755.
59. **Sarge, K. D., and E. S. Maxwell.** 1991. Intermolecular hybridization of 5S rRNA with 18S rRNA: identification of a 5′-terminally-located nucleotide sequence in mouse 5S rRNA which base-pairs with two specific complementary sequences in 18S rRNA. Biochim. Biophys. Acta **1088**:57–70.
60. **Shi, Y., and J. O. Thomas.** 1992. The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. Mol. Cell. Biol. **12**:2186–2192.
61. **Silver, P.** 1991. How proteins enter the nucleus. Cell **64**:489–497.
62. **Singh, R., and M. R. Green.** 1993. Sequence-specific binding of transfer RNA by glyceraldehyde-3-phosphate dehydrogenase. Science **259**:365–368.
63. **Stebbins-Boaz, B., and S. A. Gerbi.** 1991. Structural analysis of the peptidyl transferase region in ribosomal RNA of the eukaryote *Xenopus laevis*. J. Mol. Biol. **217**:93–112.
64. **Steitz, J. A., C. Berg, J. P. Hendrick, H. La Branche-Chabot, A. Metspalu, J. Rinke, and T. Yario.** 1988. A 5S rRNA/L5 complex is a precursor to ribosome assembly in mammalian cells. J. Cell Biol. **106**:545–556.
65. **Sterne-Marr, R., J. M. Blevitt, and L. Gerace.** 1992. O-linked glycoproteins of the nuclear pore complex interact with a cytosolic factor required for nuclear protein import. J. Cell Biol. **116**:271–280.

66. **Theunissen, O., F. Rudt, U. Guddat, H. Mentzel, and T. Pieler.** 1992. RNA and DNA binding zinc fingers in *Xenopus* TFIIIA. Cell **71:**679–690.
67. **Tobian, J. A., L. Drinkard, and M. Zasloff.** 1985. tRNA nuclear transport: defining the critical regions of human $tRNA_i^{met}$ by point mutagenesis. Cell **43:**415–422.
68. **Uchiumi, T., M. Kikuchi, and K. Ogata.** 1986. Cross-linking study on protein neighborhoods at the subunit interface of rat liver ribosomes with 2-iminothiolane. J. Biol. Chem. **261:**9663–9667.
69. **Van Ryk, D. I., Y. Lee, and R. N. Nazar.** 1992. Unbalanced ribosome assembly in *Saccharomyces cerevisiae* expressing mutant 5S rRNAs. J. Biol. Chem. **267:**16177–16181.
70. **Viel, A., M.-J. Armand, J.-C. Callen, A. Gomez de Gracia, H. Denis, and M. le Maire.** 1990. Elongation factor 1α (EF-1α) is concentrated in the balbiani body and accumulates coordinately with the ribosomes during oogenesis of *Xenopus laevis*. Dev. Biol. **141:**270–278.
71. **Westhof, E., P. Romby, P. J. Romaniuk, J.-P. Ebel, C. Ehresmann, and B. Ehresmann.** 1989. Computer modeling from solution data of spinach chloroplast and of *Xenopus laevis* somatic and oocyte 5S rRNAs. J. Mol. Biol. **207:**417–431.
72. **Wimberly, B., G. Varani, and I. Tinoco, Jr.** 1993. The conformation of loop E of eukaryotic 5S ribosomal RNA. Biochemistry **32:**1078–1087.
73. **Wormington, W. M.** 1989. Developmental expression and 5S rRNA-binding activity of *Xenopus laevis* ribosomal protein L5. Mol. Cell. Biol. **9:**5281–5288.
74. **You, Q., and P. J. Romaniuk.** 1990. The effects of disrupting 5S RNA helical structures on the binding of *Xenopus* transcription factor IIIA. Nucleic Acids Res. **18:**5055–5062.
75. **Zasloff, M.** 1983. tRNA transport from the nucleus in a eukaryotic cell: carrier-mediated translocation process. Proc. Natl. Acad. Sci. USA **80:**6436–6440.